A STUDY ON NON-CULTURE BASED TECHNIQUES IN DIAGNOSIS OF TUBERCULOUS LYMPHADENITIS

Dissertation submitted in

Partial fulfillment of the Regulations required for the award of

M.D. DEGREE

In

MICROBIOLOGY– BRANCH IV

The Tamil Nadu

DR. M.G.R. MEDICAL UNIVERSITY

Chennai

MAY 2019.

CERTIFICATE

This is to certify that the enclosed work **"A STUDY ON NON-CULTURE BASED TECHNIQUES IN DIAGNOSIS OF TUBERCULOUS LYMPHADENITIS"** submitted by **Dr.R.Pathmini** to The Tamilnadu Dr.MGR Medical University is based on bonafide cases studied and analysed by the candidate in the Department of Microbiology, Coimbatore Medical College and Hospital, Coimbatore during the period from July 2017 to May 2018 under the guidance and supervision of **Dr.P.Shankar,MD.,** Associate Professor Department of Microbiology and the conclusion reached in this study are her own.

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I, **Dr. R.Pathmini ,** solemnly declare that the dissertation entitled **"A STUDY ON NON-CULTURE BASED TECHNIQUES IN DIAGNOSIS OF TUBERCULOUS LYMPHADENITIS**" was done by me at Coimbatore Medical College Hospital, Coimbatore during the period from July 2017 to May 2018 under the guidance and supervision of **Dr. P.Shankar, M.D.,** Associate Professor, Department of Microbiology, Coimbatore Medical College, Coimbatore.

This dissertation is submitted to The Tamilnadu Dr. MGR. Medical University towards the partial fulfilment of the requirement for the award of M.D. Degree (Branch – IV) in Microbiology.

I have not submitted this dissertation on my previous occasion to any University for the award of any degree.

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Dissertation Topic	:	**A Study on non culture based techniques in diagnosis of tuberculous lymphadenitis**

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ACKNOWLEDGEMENT

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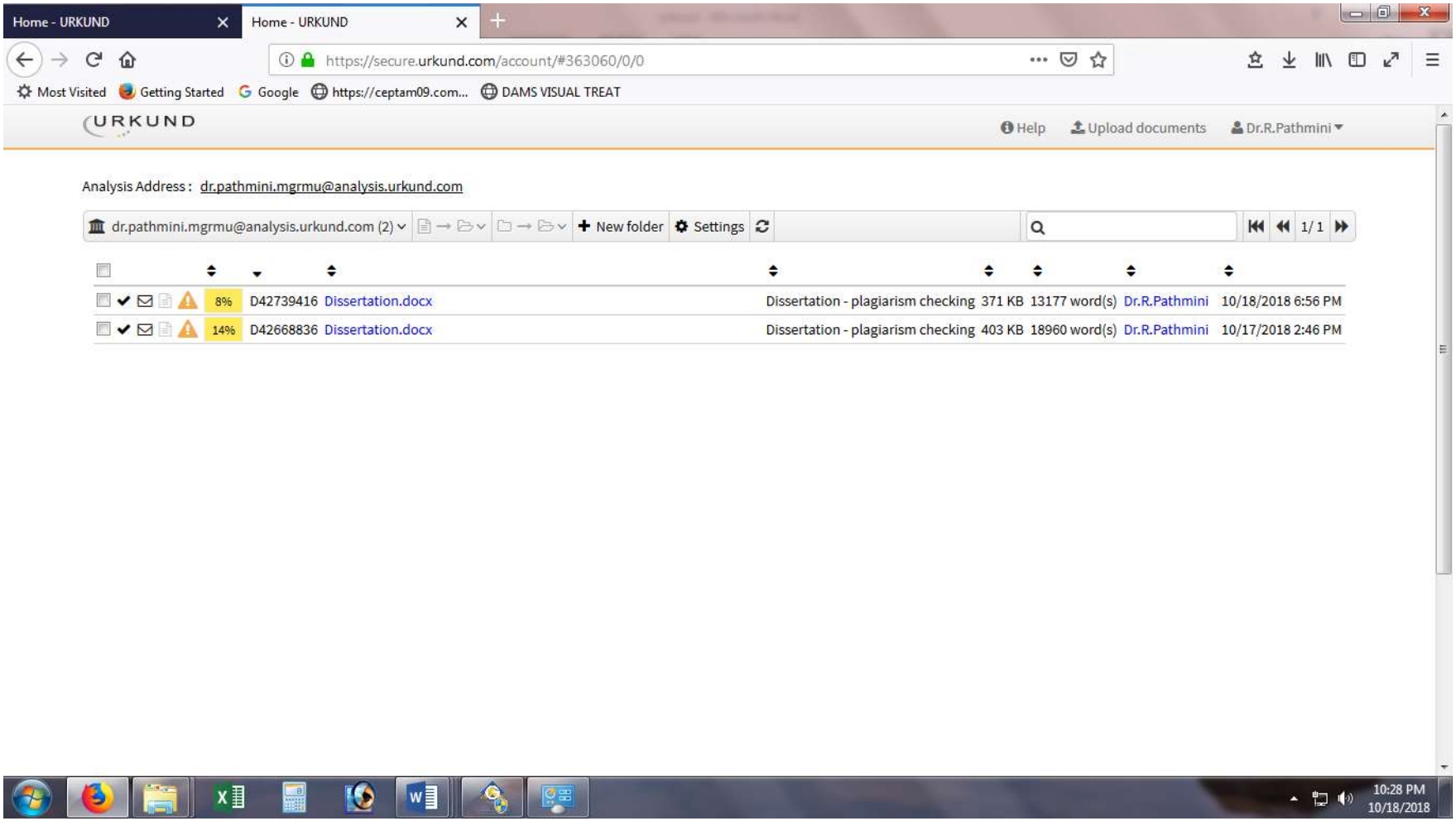
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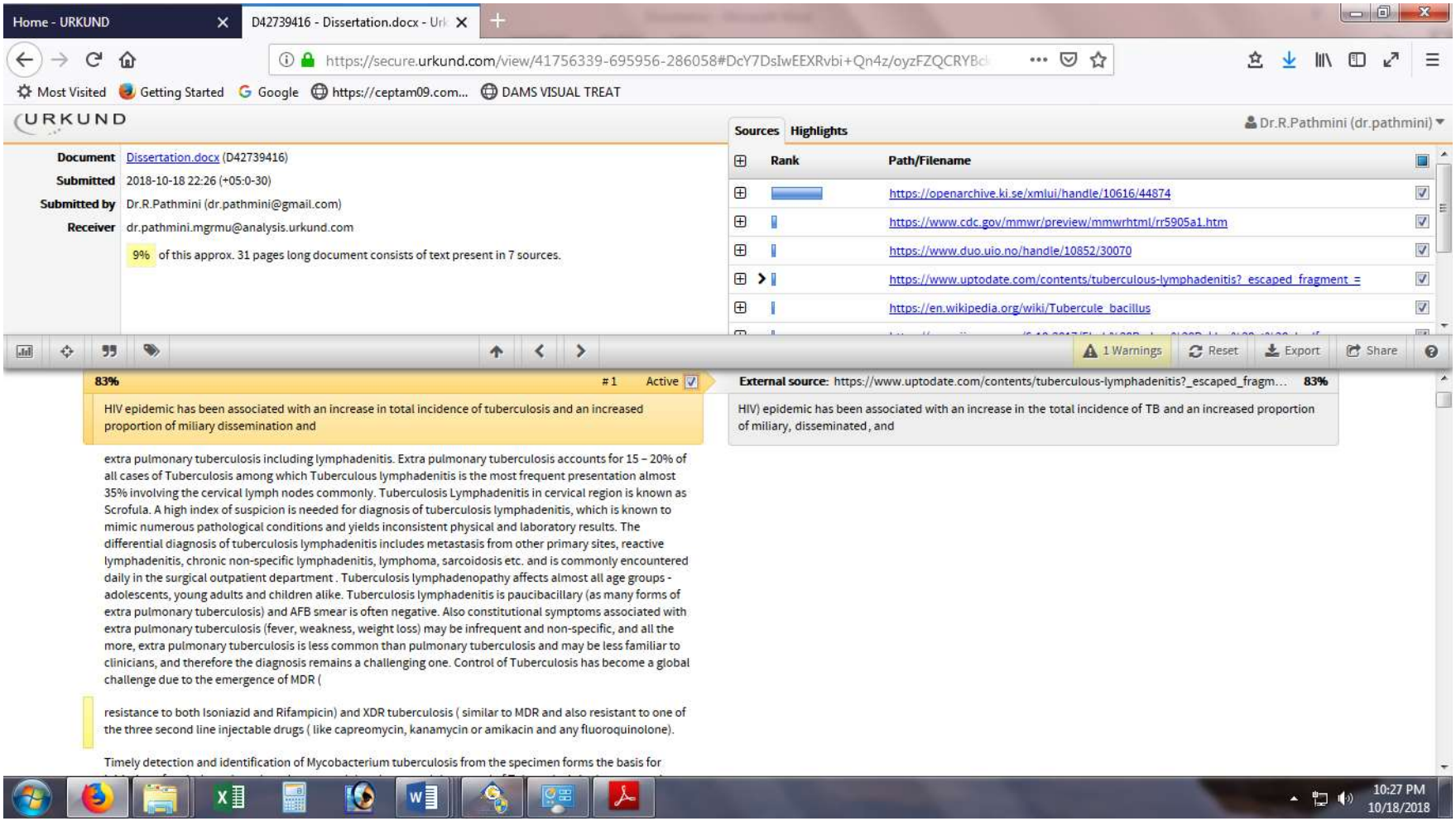
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HIV epidemic has been associated with an increase in total incidence of tuberculosis and an increased proportion of miliary dissemination and
extra pulmonary tuberculosis including lymphadenitis. Extra pulmonary tuberculosis accounts for 15 – 20% of all cases of Tuberculosis among which Tuberculous lymphadenitis is the most frequent presentation almost 35% involving the cervical lymph nodes commonly. Tuberculosis Lymphadenitis in cervical region is known as Scrofula. A high index of suspicion is needed for diagnosis of tuberculosis lymphadenitis, which is known to mimic numerous pathological conditions and yields inconsistent physical and laboratory results. The differential diagnosis of tuberculosis lymphadenitis includes metastasis from other primary sites, reactive lymphadenitis, chronic non-specific lymphadenitis, lymphoma, sarcoidosis etc. and is commonly encountered daily in the surgical outpatient department . Tuberculosis lymphadenopathy affects almost all age groups - adolescents, young adults and children alike. Tuberculosis lymphadenitis is paucibacillary (as many forms of extra pulmonary tuberculosis) and AFB smear is often negative. Also constitutional symptoms associated with extra pulmonary tuberculosis (fever, weakness, weight loss) may be infrequent and non-specific, and all the more, extra pulmonary tuberculosis is less common than pulmonary tuberculosis and may be less familiar to clinicians, and therefore the diagnosis remains a challenging one. Control of Tuberculosis has become a global challenge due to the emergence of MDR (
resistance to both Isoniazid and Rifampicin) and XDR tuberculosis (similar to MDR and also resistant to one of the three second line injectable drugs (like capreomycin, kanamycin or amikacin and any fluoroquinolone).
Timely detection and identification of Mycobacterium tuberculosis from the specimen forms the basis for
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HIV) epidemic has been associated with an increase in the total incidence of TB and an increased proportion of miliary, disseminated, and
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INTRODUCTION

A STUDY ON NON-CULTURE BASED TECHNIQUES IN DIAGNOSIS OF TUBERCULOUS LYMPHADENITIS

INTRODUCTION

Tuberculous lymphadenitis is one of the major frequent cause of lymphadenopathy and most common form of extra pulmonary tuberculosis. Tuberculosis is one of world's ancient deadliest disease. From ancient Egypt till date Tuberculosis remains the leading cause of death among notifiable infectious diseases and continues to be a disease of public health importance worldwide affecting all groups. It is a serious health problem as it ranks next to HIV infection as the top most killer globally . It has been called the 'White Plague' and "Captain of all the man of death". Emaciation often preceded a difficult death, giving tuberculosis the name, "Consumption".

With an estimation of 10.4 million new cases and 1.7 million deaths in 2016, Tuberculosis still remains a leading public health problem world wide , one-third of world's population is infected with tuberculosis. The global distribution of tuberculosis cases is skewed heavily towards low-income and emerging economics. 95% of all cases and 99% deaths due to tuberculosis occur in developing countries with the greatest burden in sub-Saharan Africa and Southeast Asia. (WHO, 2013).

India accounts for nearly $1/3^{rd}$ of global burden of tuberculosis. India has the highest number of tuberculosis cases in the world, with over 2 million cases every year , accounting for $1/4^{th}$ of the global incidence

of Tuberculosis . It is estimated that about 40% of Indian population is infected and the prevalence of tuberculosis in India was estimated to be 249/1,00,000 population with mortality of 23/1,00,000 (11). Poverty and Tuberculosis go together. Tuberculosis had declined rapidly in the affluent nations with improvements in standards of living and the use of effective Anti-tuberculous therapy. But with the progress of the AIDS epidemic, Tuberculosis especially extra pulmonary presentation is a problem for even the rich nations also. The HIV epidemic has been associated with an increase in total incidence of tuberculosis and an increased proportion of miliary dissemination and extra pulmonary tuberculosis including lymphadenitis.

Extra pulmonary tuberculosis accounts for 15 – 20% of all cases of Tuberculosis among which Tuberculous lymphadenitis is the most frequent presentation almost 35% involving the cervical lymph nodes commonly. Tuberculosis Lymphadenitis in cervical region is known as Scrofula.

A high index of suspicion is needed for diagnosis of tuberculosis lymphadenitis, which is known to mimic numerous pathological conditions and yields inconsistent physical and laboratory results. The differential diagnosis of tuberculosis lymphadenitis includes metastasis from other primary sites, reactive lymphadenitis, chronic non-specific lymphadenitis, lymphoma, sarcoidosis etc. and is commonly encountered daily in the surgical outpatient department . Tuberculosis lymphadenopathy affects almost all age groups - adolescents, young adults and children alike.

Tuberculosis lymphadenitis is paucibacillary (as many forms of extra pulmonary tuberculosis) and AFB smear is often negative. Also constitutional symptoms associated with extra pulmonary tuberculosis (fever, weakness, weight loss) may be infrequent and non-specific, and all the more, extra pulmonary tuberculosis is less common than pulmonary tuberculosis and may be less familiar to clinicians, and therefore the diagnosis remains a challenging one.

Control of Tuberculosis has become a global challenge due to the emergence of MDR (resistance to both Isoniazid and Rifampicin) and XDR tuberculosis (similar to MDR and also resistant to one of the three second line injectable drugs (like capreomycin, kanamycin or amikacin and any fluoroquinolone). Timely detection and identification of Mycobacterium tuberculosis from the specimen forms the basis for initiation of antituberculous drug therapy and thereby control the spread of Tuberculosis in the community. Though the bedside decision on the initiation of anti tuberculous drugs therapy is based on epidemiologic, clinic, radiographic and or histological findings, it should be further supported by atleast a rapid microbiological test or positive AFB smear result.

Tuberculosis is diagnosed by demonstration of Mycobacterium tuberculosis in suitable clinical samples collected from patients, whereas other clinical investigations may suggest the diagnosis of Tuberculosis, but cannot confirm the diagnosis. The control of tuberculosis is hampered

by diagnostic methods with suboptimal sensitivity and specificity. Early diagnosis is one of the primary challenges in curtailing its spread.

Usually tuberculosis lymphadenitis presents as a slowly progressive, painless swelling of a single group of lymph nodes. The duration of symptoms at the time of presentation is typically 1-2 months, varying from 3 weeks to 8 months. The mean duration of symptoms is longer in men than in women. Though microscopy is simple and cheap, it has low sensitivity and specificity, and culture using Lowenstein Jensen media though more sensitive than microscopy and also highly specific ,delays diagnosis by 3 – 8 weeks.

Liquid culture system though rapid and more sensitive and specific than Lowenstein Jensen, are very expensive. Since conventional techniques have limitations in early and rapid tuberculosis diagnosis, fine needle aspiration and PCR will be instrumental in arriving at an early diagnosis for early institution of therapy before a final diagnosis can be made by biopsy and culture.

Molecular methods like polymerase chain reaction (PCR) increases the diagnostic accuracy when the cytology / AFB smear fails to detect tuberculosis and avoids the need for open biopsy of cervical nodes. Nucleic acid amplifications methods targeting mycobacterium tuberculosis sequence in clinical specimens are increasingly in use as a tool for early diagnosis of tuberculosis. The Insertion sequence of IS6110 is a mobile genetic element and has universal acceptance, since it is found only in

mycobacterium tuberculosis complex and also the strain carries multiple copies of the element.

The reversal of steady decline in tuberculosis in the developed and the parts of developing countries in the last 10 years is a major concern. Case detection rate of TB has been estimated in only 64% and remaining 36% of the incident TB cases are not detected (WHO). Approximately 3.3 million cases are missed throughout the world .

Delay in the diagnosis and treatment increases the severity of the disease and is associated with higher risk of mortality and morbidity more so in HIV infected patients. This delay is also an important contributor for increased nosocomial outbreaks among patients and health care workers. Hence early diagnosis of tuberculosis is crucial for prompt treatment and for the control of disease transmission.

AIM & OBJECTIVES

AIM AND OBJECTIVES

AIM:

1) To study the epidemiology of tuberculous lymphadenitis from samples obtained by FNAC.
2) To process the sample by non culture techniques by AFB smear study.
3) To detect the MTB specific genes using RT –PCR and GeneXpert.

OBJECTIVES

1) To compare the cytomorphological presentations by haematoxylin and eosin stain and AFB by Ziehl Neelsen using FNA in clinically suspected cases of tuberculous lymphadenitis.
2) To amplify the MTB specific gene - IS6110 by RT-PCR.
3) To detect the gene mutations coding for drug resistance .

REVIEW OF LITERATURE

REVIEW OF LITERATURE

TUBERCULOUS LYMPHADENITIS

Tuberculous lymphadenitis is a chronic specific granulomatous inflammation of lymph nodes with caseous necrosis caused by the Mycobacterium tuberculosis complex. It is the most common extra pulmonary form and the oldest disease seen in Hippocrates writing dating back to 460-377 BC.

Tuberculous lymphadenitis may arise without a preceding pulmonary involvement constituting 28% to 80% of 1/3rd of tuberculosis disease [9].

Diagnosis may be challenging as clinical manifestations are variable from that of pulmonary tuberculosis and response to therapy is slow or paradoxical with enlarging or new lymphnodes. If untreated the disease may be fatal within 5 years in 50-65% of cases.

HISTORY

Hippocrates, in book 1 of his “OF THE EPIDEMICS”, describes the characteristics of the disease. Franciscus Sylvius was the first person to recognise the skin ulcers caused by Scrofula resemble tubercles in 1679. Rene Laennec who invented stethoscope died at the age of 45 due to tuberculosis. In 1882, 24th march Robert Koch, a Prussian physician discovered tubercle bacilli utilizing new stain. He succeeded in cultivating the bacillus on inspissated serum and transmitted the disease to many animals of different species by inoculation with pure culture of the bacillus. This became the basis for Koch's postulates. In 1895 Roentgen

discovered the X-Ray which helps in diagnosing and tracking the progression of the disease. In 1908 Charles Mantoux found PPD developed by Koch is effective for intradermal test in diagnosing tuberculosis. The BCG (Bacille – Calmette Guerin) vaccine developed in 1906. It was first used on humans in1921 in France.

The scientific understanding of tuberculosis and its contagious nature created the need for institution to house sufferers and was established in Germany in 1854.

In 1944, streptomycin was discovered by Elizabeth Bugie and it was the first antibiotic against M.tuberculosis.

In 1952, Isoniazid, the first oral mycobactericidal drug was discovered. The advent of rifampicin in 1970s hastened recovery and significantly reduced the number of cases until 1980s.

ETIOLOGIC AGENT

Mycobacteria belong to the family mycobacteriaceae and the order Actinomycetales. The name of the genus Mycobacterium given by Lehmann and Neumann in 1896 for mould like pellicles in liquid media (Greenwood)

Cell Structure

The cell wall contains peptidoglycan layers with branched chain polysaccharides, proteins and lipids. Long chain mycolic acid and lipoarabinomannan (LAM), a lipid polysaccharide complex contributes to unusually high lipid content (>60% of total cell wall mass) makes them

hardly, impenetrable and hydrophobic . It is stained only with heat , prolonged time and penetrating agent. Electron microscopy shows a bilayered cell membrane with mesosomes. The cytoplasm contains polyphosphate granules , a nuclear body, electron translucent vacuoles probably responsible for beaded appearance.

Staining Reaction

Mycobacteria are difficult to stain. In a Gram stained smear, they appear either as negatively stained images or "ghosts", or beaded Gram positive rod, or they may be Gram invisible. The presence of Gram ghost could provide an early clue to the presence of Mycobacteria.

Acid fast staining

When Koch isolated the tubercle bacilli, he used alkaline methylene blue for 24 hrs and decolorized them with Bismarck brown. Ehrlich stained the organism with fuchsin and aniline oil as mordant and mineral acid as decolouriser. Ziehl changed the mordant to carbolic acid. Neelsen (1883) increased the strength of carbolic acid and used dye to make carbol fuchsin . Ziehl Neelsen (ZN) method stains Mycobacteria in red colour of carbol fuchsin even after decolourisation with mineral acid and hence are called acid fast due to the formation of a complex of dye within the cell.

Kinyoun developed a "cold" acid-fast technique in which instead of heat , concentration of carbolic acid and fuchsin was increased.

Nutritional / Growth requirement

The basic nutritional requirements of Mycobacteria for in vitro growth are oxygen , carbon, nitrogen, iron , phosphorus, sulphur, magnesium and various trace elements.

Robert Koch made the first successful attempt of cultivation of Mycobacterium tuberculosis in 1882.Widely used media for cultivation of Mycobacteria contain egg (coagulated) or agar as solidifying agent.

Resistance:

Physical Agents:

Mycobacteria are resistant to drying if protected from sunlight. It is sensitive to ultraviolet light and depends on pigment content. Scotochromogenic species are resistant and photochromogenic strains are most sensitive. At room temperature cultures can be maintained for 6-8 months . In deep freeze, Mycobacteria survive for about 2 years when stored at -20ºC. Pasteurization of milk kills the Mycobacteria as they get killed at 60ºC in 20 minutes .

Chemical Agent:

The acid-fast bacilli have a high degree of resistance to 15% suplhuric acid, 3% Nitric acid 4% sodium hydroxide. Mycobacteria are sensitive to 2% phenol 3% formalin, 10% sodium hypochloride solution, formaldehyde, ethylene oxide, and 70-80% ethanol. Mycobacteria are rapidly killed by acetone, propenol.

Antigens:

The Mycobacterial antigens are broadly classified:

1. According to their chemical structure as carbohydrate, lipid and protein
2. As Cytoplasmic (soluble) or cell wall lipid bound (insoluble)
3. According to their distribution within the genus.

Polysaccharides:

The main immunogenic polysaccharides are arabinomannans and arabinogalactans which are linked to mycolic acid in the cellwall.

Lipids:

The important lipid antigens are sulphatides / sulpholipids and wax D. Sulpholipid prevents degradation of bacilli by inhibiting fusion of lysosome with phagosome.

Cord factor:

Consists of two mycolic acids linked to the trehalose and was responsible for serpentine cord-like growth of the Mycobacteria in liquid media. It induces granuloma formation and provokes an acute inflammatory process by activating alternative complement pathway .

Wax D:

It gives shape and rigidity to the cell and composed of mycolic acid attached to murein by arabinogalactan .

Mycosides:

Mycosides are surface lipids which plays important role in pathogenesis and serves as receptor sites for phages.

Proteins:

Protein antigens located in the cytoplasm proved useful for qualitative assays in identification of tubercle bacilli

Stress Protein:

As Mycobacteria enters the phagocytic cell, a number of stress proteins are released .

Tuberculin:

It is the earliest preparation of Mycobacterial antigens used by Koch for experiment. A skin test with purified protein derivative may be used — (1) for diagnosis of active infection in infants and young children, (2) to measure the prevalence of infection in the community, (3) to select susceptible population for BCG vaccination.

EPIDEMIOLOGY

Studies on epidemiology of tuberculosis are concerned with the transmission of the disease in the community and the impact of the control measures. Extra pulmonary tuberculosis accounts for 15 – 20% of all cases of Tuberculosis(3). Tuberculous lymphadenitis is the most frequent presentation of extra pulmonary tuberculosis which accounts for 35% and most commonly cervical lymph nodes are affected. One-third of world's population is infected with tuberculosis. In 2016, 10.4 million people around the world became sick with tuberculosis disease. There were 1.7 million tuberculosis-related deaths worldwide (CDC).

India accounts for nearly 1/3rd of global burden of tuberculosis. India has the highest number of tuberculosis cases in the world, with over 2 million tuberculosis cases every year. Annually, 1/4th of the global incident tuberculosis cases occur in India.

In Tamil Nadu, 3176 paediatric patients were notified and total number of tuberculosis patients notified was 96,079. Here more alarming was notified cases with known HIV status were 94,157. The Incidence in Tamil Nadu is 67,585. Number of TB patients known to be HIV positive was 4708.(1)

Most affected states in India are Uttar Pradesh, Madhya Pradesh, Maharashtra, West Bengal, Rajasthan, Bihar , Gujarat , Andhra Pradesh, and Tamil Nadu.

People at high risk of exposure to infection with M.tuberculosis

- Contacts of people with known or suspected to have TB disease and infants, children, and adolescents are more prone to develop LTBI/TB disease.
- People who have come from endemic areas within the last 5 years (for example Asia, Africa, Russia, Eastern Europe, or Latin America).
- People who visit areas with a high prevalence of TB disease.
- People who live or work in high-risk congregate settings (for example, nursing homes, homeless shelters, or correctional facilities).
- Health care workers

People at high risk for developing TB disease after infection with M.tuberculosis

- People living with HIV.
- Children younger than 5 years of age.
- People recently infected with M.tuberculosis (within the past 2 years).
- People with a history of untreated or inadequately treated TB disease.
- Persons who are receiving immunosuppressive therapy
- Persons with silicosis, diabetes mellitus, chronic renal failure, leukemia, or cancer of the head, neck, or lung.
- Persons who have had a gastrectomy or jejunoileal bypass.
- Low body weight.
- Cigarette smokers and persons who abuse drugs or alcohol.

One of the most threatening features of tuberculosis in HIV infected patients has been the spread of multidrug resistant (MDR) organisms. The national AIDS policy documents from Government of India show that >60% of AIDS patients suffer from tuberculosis as an opportunistic infection.(50).

Tuberculosis Resurgence

In 1980s, due to emergence of drug resistant strains and emergence of HIV epidemic there was a resurgence following which WHO issued a declaration of a global health emergency in 1993. As a response to global emergency ,WHO developed DOTS (Directly Observed Therapy Short course) and implemented in 184 countries. Target by 2005 was achieved for treatment success(83%) but not for case detection (only 53%). In view of improving case detection rate, WHO shifted from DOTS to TO STOP TB partnership strategy in 2006 which includes Practical Approach to Lung health (PAL) to improve case detection.

SOURCE CASE

1. Bacillary population
2. Aerosol generation
3. Consumption of raw milk from infected cows
4. Poorly heat treated meat
5. Closer contact with infected animals.

↓

EXPOSURE TO CONTACTS

1. Intensity
2. Duration

↓

CONTACTS

1. Inborn defenses
2. Immunologic defenses

↙ ↘

No Infection (50%)

Infection (25 – 50%)
Cell-mediated immunity

↙ ↘

No disease (90%)

Disease (10%)

↙ ↘

"Early"
Progressive (5%)

"Late"
Recrudescent (5%)

It is well established that, sputum positive patients i.e. positive on direct microbiological examination and thus contains at least 10,000 bacilli per 1 ml and who has either received no treatment or not being treated fully, can discharge the bacilli in their sputum for years. They become the potential sources of infection.

TRANSMISSION AND DEVELOPMENT OF DISEASE

Almost all M.tuberculosis infection is acquired by the inhalation of aerosolized droplet nuclei (1-5·µm) caused by coughing, sneezing, speaking and singing. There may be 3000 infectious nuclei per cough. Droplet nuclei in the range of 1 to 10µm can be inhaled, with the larger ones trapped in the upper nasal passages or expelled into the pharynx by the mucociliary mechanism of the lower respiratory tract and harmlessly swallowed and digested; smaller droplet nuclei may reach the alveoli and establish infection. Transmission through tonsil and GIT occurs from ingestion of raw milk. Crowding is most important factor in transmission of tubercle bacilli .

IMMUNE RESPONSE

Infection with M. tuberculosis provokes both cell mediated as well as humoral immune response. However, only cell-mediated immunity is found to be a protective mechanism in Tuberculosis. The initial immune response in a non-immune individual is a nonspecific inflammatory reaction. The macrophage plays a key role in processing the bacterial antigen and present it to the appropriate lymphocytes which results in activation of macrophage by Interferon gamma.

The activated macrophage kills the phagocytosed organism with the help of reactive oxygen radical and reactive nitrogen intermediate including NO, NO2 and HNO3.

Granulomatous reaction

This is a type of delayed hypersensitivity shown by sensitized CD4+T cells in response to non- degradable or persisting antigens. The granuloma formation is necessary for separation of infecting organism from the rest of the tissue.

The sensitized TH1 cells secrete IL-2 which stimulate cytotoxic CD8+ T-cell. These cells kill the antigen bearing macrophage. On lysis of macrophage some tubercle bacilli are set free.

Tuberculin type of delayed hypersensitivity

In a sensitized individual intradermal injection of soluble antigen elicits the delayed hypersensitivity state of a short duration and even the dermal response is more transient. This is known as 'Jones-Mote' sensitivity but recently been termed as "Cutaneous basophil hypersensitivity" because of high infiltration of basophils in the skin lesion.

MYCOBACTERIUM TUBERCULOSIS COMPLEX

The term complex describes 2 or more species for which distinction complicated and has little or no medical importance and differentiation for epidemiology and public health reasons. M.tuberculosis complex include;

1. M.tuberculosis

2. M.bovis & M.bovis BC
3. M.africanum
4. M.caprae
5. M.microti
6. M.canetti
7. M.pinnipedic
8. M.mungi
9. M.orygis

They are slow growers and colonies are non-pigmented. Tuberculosis is caused by bacteria belonging to the M. tuberculosis complex, which consists of highly related slow growing, acid-fast, aerobic, non-spore forming, non-motile bacteria. They form slightly curved or straight rods which may branch (0.2 to 0.6 μm by 1.0 to 10 μm) (48).

Mycobacterium tuberculosis

M. tuberculosis is the principal agent of TB in humans. Phenotypically, M. tuberculosis can be identified using analysis such as nitrate reductase, production of niacin, resistance to thiophene-2-carboxylic acid hydrazide (TCH) and sensitivity to pyrazinamidase (55,56). Genotypically, by Spacer Oligonucleotide Typing (spoligotyping), it is classified into different phylogenetic lineages (57).

Mycobacterium bovis and Mycobacterium bovis BCG

Bovine Tuberculosis, caused by M. bovis is the main zoonotic disease caused by mycobacteria, affecting cattle, other domesticated

animals and certain free or captive wildlife species. The disease is spread to humans, typically by ingestion of unpasteurized milk or contaminated meat, causing extrapulmonary Tuberculosis, but can also be transmitted by inhalation of aerosols causing pulmonary Tuberculosis (58,59)

There is also a non-virulent strain of M. bovis called Bacillus Calmette Guerin (BCG), which has its origin from a virulent M. bovis strain (64). It is used worldwide as a live attenuated vaccine to immunize people against TB.

Mycobacterium africanum

M. africanum is phenotypically heterogeneous, with characteristics common to both M. tuberculosis and M. bovis. Based on their geographic origin and biochemical characteristics, two subgroups of M. africanum have been described, in western Africa (subtype I) and eastern Africa (subtype II) (73).

Mycobacterium canettii

M. canettii, a rare variant of the M. tuberculosis complex with smooth colony morphology, it differs from the other strains by having large amounts of lipooligosaccharides on the cell wall (75).

Mycobacterium microti

M. microti, is the causative agent of TB in voles, wood mice, and shrews and can also cause disease in a limited number of other mammalian species. M. microti strains display characteristic Insertion sequence (IS)6110 Restriction Fragment Length Polymorphism (RFLP) banding patterns and spoligotypes, distinct from other M. tuberculosis complex strains (77).

Mycobacterium pinnipedii

It is first isolated in seals.Transmission of M. pinnipedii to humans has been reported in individuals who are in close contact with marine mammals (62,63).

M. pinnipedii isolates present a distinct spoligotype pattern when compared to other members of the M. tuberculosis complex (79).

1.4.7 Mycobacterium caprae

M. caprae was first isolated from goats. Based on biochemical tests, results are similar to M. bovis and M. bovis BCG.

Novel variants of the M. tuberculosis complex M. mungi, was identified as the causative agent of TB in banded mongooses. M. mungi was characterizes as highly virulent, causing high numbers of deaths in a short period of time (2–3 months from clinical presentation to death), apparently through environmental transmission (nonrespiratory route) (71).

TUBERCULOUS LYMPHADENITIS

Tuberculosis of the superficial lymph nodes is the commonest extra pulmonary manifestation of the disease. In India, approximately 15-20% of all tuberculosis cases is extra pulmonary and of these 2/3rd are due to lymph node disease, the commonest site being cervical lymph nodes.

Pathophysiology

Tuberculosis of lymph node occurs;

1) Either as a result of primary infection,

2) As reactivation of previously contained foci or

3) By extension from a contiguous focus

In primary infection, most commonly acquired by airborne infection, non-specific inflammatory reactions occur as the mycobacteria reach the alveoli , and phagocytosis and intra cellular replication takes place. A bacteremia occurs with dissemination throughout the body. Lymphatic involvement is an integral part of tuberculous infection with generalized lymphatic and hematogenous spread rather than a localized disease process.

HIV Infection And Extra Pulmonary Tuberculosis

Extrapulmonary disease has been reported in up to 70% of HIV related tuberculosis cases when the CD4 lymphocyte count is less than 100(36). HIV related tuberculosis lymphadenopathy could occasionally be acute and resemble an acute pyogenic bacterial infection. .

CD4 cell mediated immunity and macrophage function are essential in the control of M.tuberculosis infection. The hallmark of HIV infection is progressive deterioration and depletion of CD4 cells, coupled with defects in macrophage and monocyte function and increasing tissue destruction. The risk of tuberculosis developing in a HIV infected a patient who is latently coinfected with MTB approaches 10% per year, as opposed to a 10% lifetime risk in an immuno competent host.

Clinical Features

Persons affected with tuberculous lymphadenitis may have nonspecific constitutional symptoms(4). Locally, there may be painless

swelling with or without a discharging sinus or a scar or both. A classical presentation is multiple, matted lymph nodes with variable consistancy which are painless and slow growing.

The physical appearance of superficial tuberculous lymphadenitis has been classified into five stages by Jones & Campbell.

Stage 1 - Enlarged, firm, mobile, discrete nodes, slightly tender. Histologically they show non-specific reactive hyperplasia.

Stage 2 - Large, rubbery nodes fixed to surrounding tissue getting adherent to each other called as matting. Histologically they show periadenitis, typical tuberculous granulomatous tissue with lymphocytes, epithelioid cells and caesation.

Stage 3 - Central softening due to abscess formation called cold abscesses because of extensive caesation.

Stage 4 – The abscesses burst out of lymph node mass and extends into subcutaneous tissues giving raise to Collar stud. Abscess.

Stage 5 – The abscesses burst and persistent discharging sinus tract formed.

Tuberculosis lymphadenitis is a common disease of children. However in the recent past, there is a peak age range of 20-40 years. Tuberculous lymphadenitis in adults show striking female preponderance.

DIAGNOSIS OF TUBERCULOUS LYMPHADENITIS

Tuberculous lymphadenitis often presents a diagnostic challenge especially when clinical presentation is suggestive but bacteriological proof is lacking.

The differential diagnoses of tuberculous lymphadenitis are many:

1. Neoplasia - Hodgkin's and Non Hodgkin's Lymphoma, sarcoma.
2. Other infection-acute bacterial infection, viral infection, (infectious mononucleosis), Chlamydia, toxoplasmosis, fungal infection, nontuberculous, mycobacterial infection, BCG adenitis.
3. Drug reaction - eg. Hydantoin
4. Sarcoidosis
5. Non-lymphode swelling - Submandibular or parotid glands, bronchial cysts, cystic hygroma, carotid body tumour, thyroid swellings.

Histological Diagnosis

Four distinct types of histological features are seen in lymphnode biopsies(33). Most common is the 'reactive' type with typical tuberculous granuloma, with fine eosinophilic caseation necrosis, epithelioid cells, giant cells, plasma cells and lymphocytes. Next in frequency is the 'hyperplastic' type with well-differentiated epithelioid cell granuloma with very little necrosis. The third is 'hyporeactive' type with poorly organized granuloma with macrophages, immature epitheloid cells, lymphocytes, plasma cells and coarse predominantly basophilic caseation necrosis and 4th type is a nonreactive type unorganized granuloma with granuloma with macrophages, lymphocyte, plasma cells & polymorphs with non caseating necrosis.

Cytological Diagnosis

This simple outpatient procedure has provided an alternative and an easy method for collection of material for cytomorphologic and bacteriologic examination.

Using one of the various techniques using stains such as Haematoxylin-Eosin stain, Giemsa stain, wright stain and papanicolaou stain,the cytology is studied.

The disadvantage is that it involves interpretation of limited sample obtained by the aspiration. The aspiration technique is of at most importance. Even in the hands of an experienced person the percentage of 'Nonrepresentative' aspirates may be up to 20% or more. It is difficult to exactly localize and aspirate the small and deep-seated lesions.

Bacteriological Diagnosis

Lymph node tuberculosis, like other form of extra pulmonary disease is a paucibacillary condition. The yields of positive cultures and AFB smear study from the lymphnodes have not been high.

LABORTORY DIAGNOSIS OF TUBERCULOUS LYMPHADENITIS

This can be broadly divided into

1) Demonstration by Direct microscopy - light and Fluorescent
2) Isolation of mycobacterium tuberculosis by culture
3) Serodiagnosis of the infection
4) Molecular methods

Direct Microscopy -Staining

i) Ziehl-Neelsen Staining (ZN)

ZN staining is a highly specific technique where AFB is demonstrated . It is positive only if number of AFB is more 10000 per ml of specimen(19).

ii) Speical Staining For AFB

Fluorescent Microscopy

In this technic , Auramine phenol or Auramine Rhodamine were used. Brightly fluorescent bacilli appear Yellow against a dark background(10). The advantages of fluorescent microscopy are 1) the method is more sensitive than ordinary microscopy, 2)since organism can be observed at low power, slides can be screened more rapidly than Bright field microscopy. 3) It is useful in following the progress of known tuberculous patients .4) It helps to identify the source of infection in community.

Mycobacterial Culture

Definitive diagnosis depends on the bacteriological isolation and identification of Mycobacterium species from clinical specimens or molecular study of strain.

Specimen Preparation:

Homogenization:

N-acetyl -L Cysteine (NALC) or 4% Sodium Hydroxide (NaOH) is used as a mucolytic agent to assist liquefaction . of sputum and purulent

samples(41).

Digestion and decontamination:

Various agents such as 4% sodium hydroxide, trisodium phosphate alone or in combination with benzalkonium chloride (Zepheran), N-Acetyl L cysteine (NALC), cetyl pyridium chloride (CPC),4% oxalic acid and 5% sulphuric acid can be used.

Neutralization :

eutralized by the addition of distilled water or phosphate buffer, in case of decontamination with NALC.

Centrifugation :

This centrifugation is necessary to concentrate the bacilli. The recovery of culture is increased when the relative centrifugal force is around 3000g for 15 minutes.

Inoculation of specimens :

Some of the non-selective media available are Lowenstein Jensen medium which is most commonly used in most of the clinical diagnostic laboratories.

Some of the selective mycobacterial isolation media are Gruft's modification of Lowenstein Jensen, selective 7H11 (Mitchinson) medium, Middle brook 7H 10 medium. For extra pulmonary tuberculosis, the selective Kirchner synthetic liquid medium containing horse or bovine serum is used.

Automated Detection Systems:

Many Semi-automated and automated mycobacteria detection systems are currently available; they are the radiometric Bactec system, non-radiometric MB/Bac T System and ESP Myco system. Other manual systems are the mycobacteria Growth Indicator Tube (MGIT) 13 system and Septi-chek System

The BBL™ MGIT™ Mycobacteria Growth Indicator Tube contains 7 mL of modified Middlebrook 7H9 Broth base. The complete medium, with BACTEC™ MGIT™ 960 Growth Supplement and BBL™ MGIT™ PANTA™ Antimicrobic mixture, is one of the most commonly used liquid medium for the cultivation of mycobacteria.

The BACTEC™ MGIT™ 960 System is designed for the rapid detection of mycobacteria in all types of clinical specimens except blood and urine. The system includes a liquid culture medium (BBL™ MGIT™ Mycobacteria Growth Indicator Tube), a growth supplement and an antibiotic mixture (BBL™ MGIT™ PANTA™). The BACTEC™ MGIT™ Growth Supplement provides substances essential for the growth of mycobacteria. BBL MGIT PANTA contains a mixture of antimicrobial agents used to suppress the growth of contaminating bacteria.

A fluorescent compound is embedded in silicone on the bottom of each of the MGIT broth tubes. This compound is sensitive to the presence of oxygen dissolved in the broth. Initially, the large amount of dissolved oxygen quenches the emissions from the compound and little fluorescence

can be detected. Later, actively respiring microorganisms consume the oxygen and allow the fluorescence to be detected.

IDENTIFICTION OF M. TUBERCULOSIS

Species level identification of Non-tuberculous mycobacterium Runyon's Classification

Runyon Group	**Growth Rate**	**Pigmentation**	**Typical Member**
I	Slow > = 5 day	Photochromogenic (Yellow or orange Pigment after exposure to light)	M. Kansasi M. marinum
II	Slow > 5 days	Scotochromogenic (Yellow or orange pigment in the dark)	M. scrofulaceum
III	Slow > 5 days	Nonchromogenic No pigment in the dark or in the light	M. avium M. intracellulare
IV	Rapid < 4 days	Variable	M.fortuitum complex M. smegmatis M. phelei

PHENOTYPIC APPROACHES FOR IDENTIFICATION OF MYCOBACTERIA

Pigment Production Test

On the basis of carotenoid pigment production, mycobacteria are classified into three groups including: Photochromogenic [producing pigments in the light], Scotochromogenic [producing pigments in the dark], and non-chromogenic.

Growth Rate Calculation

Mycobacteria are divided into two groups based on their growth rate: slow growing and rapidly growing. In order to calculate growth rates

of mycobacteria, a special culture medium named N-Medium is used and is placed in incubators for as long as 18 days. As a rule of thumb, slow-growing mycobacteria cause turbidity in culture medium in more than 7 days, while rapidly growing strains do the same in less than 7 days (75).

Niacin Reduction Test

M. simiae and M. tuberculosis lack the enzyme for conversion of niacin to ribonucleotides and helps in identification of these species.

Nitrate Reduction Test

M. tuberculosis are able to reduce nitrate to nitrite. The nitrate reduction test is performed for the diagnosis of M. tuberculosis, M. kansasii, M. szulgai and also some non-pathogenic photochromic stains. M. fortuitum is also nitrate positive. M. avium, M. xenopi, M. simiae and M .marinum are all nitrate negative or hardly positive (75).

Catalase Test

Catalase is a soluble intracellular enzyme which breaks hydrogen peroxide into water and oxygen.

Semi-quantitative Catalase Test

M. gastri, M. avium, M. marnium, M. tuberculosis, M. haemophilum and M. bovis produce a bubble column with a height less than 45 mm while the other species make higher columns.

68 degree Celsius Catalase Test

The test is positive for only a few of mycobacteria which their catalase enzyme can resist to being heated at 68 degree Celsius for 20 minutes (76).

Iron Uptake Test

Rapidly growing mycobacteria like M. furtitum and M. fline that can absorb iron salts form the culture environment (75).

Potassium Tellurite Reduction Test

Reduction of colorless potassium tellurite to metallic black tellurite in the course of 3 to 4 days, is a determinant of presence of M. avium complex and other rapidly growing mycobacteria (76).

Tween 80 Hydrolysis Test

Enzymatic hydrolysis of tween 80 by mycobacteria results in colour change of the substrate and useful in the diagnosis of scotochromogenic and non-chromogenic mycobacteria (76).

Arylsulfatase Test

Determining arylsulfatase enzyme activity is useful in differentiating fast growing mycobacteria from non-Photochromogenic ones (75).

SEROLOGICAL DIAGNOSIS

Sensitivity and specificity increase if ELISA with purified antigen is done. The antigens tested in serological assays include the 38 KDa antigen, Lipoarabinomannan, Antigen-60(32), antigen 85 KDa complex and glycolipids including phenolic glycolipid Tb1, 2y3 – diacyl trehalose and lipooligosaccharide.

Most patients with tuberculosis produce antibody to glycolipids and 38 KDa and 85 complex antigens . A number of antigen capture assays based on enzyme-linked immunosorbant assay, western blot analysis of

M. tuberculosis H37RV culture filtrates antigen, radio immunoassay or agglutination of antibody coated latex particles have been described.

Immunochromatographic identification of the M. tuberculosis complex

The immunochromatographic assays, also called lateral flow assays, allows differentiation between the M. tuberculosis complex and NTM. It uses a monoclonal antibody to detect the MPB64 protein (Rv1980c; also termed as MPT64), which is secreted by M. tuberculosis complex bacteria. The immunogenic protein MPB64 is highly specific for M. tuberculosis complex, except some variants of M. bovis BCG (75).

Detection of lipoarabinomannan (LAM`) in urine sample

A number of mycobacterial antigens can be detected in the urine of patients with pulmonary TB, but the most promising of these to emerge is the cell wall lipopolysaccharide lipoarabinomannan (LAM`) (75).

Antigen-Protein Detection

M. tuberculosis appears to be the only one of these species that releases tuberculostearic acid and the presence of this substance in CSF is thought to be diagnostic of tuberculous meningitis .

Production of adenosine deaminase, a host enzyme, is increased in certain infections caused by M. tuberculosis.

Immunodiagnostic Testing

As previously discussed, interferon-gamma release assays have become more widely used for the diagnosis of tuberculosis.

MOLECULAR APPROACHES FOR TB DIAGNOSIS

THE IMPORTANCE OF STUDIES ON MOLECULAR EPIDEMIOLOGY OF TB

Below are summarized some applications of molecular techniques in TB epidemiology described by Mathema and colleagues (47):

- Study of the M. tuberculosis complex transmission dynamics (outbreak, transmission, chains of transmission, risk factors and groups at risk of M. tuberculosis complex infection).
- Discriminating recurrent TB due to exogenous reinfection and reactivation.
- Detection of laboratory error/cross-contamination.
- Determination of geographic spread of strains.
- Monitoring transmission of drug-resistant strains.
- Investigation of the evolution of drug-resistant TB within and between patients.
- Detection of mixed infections among TB patients.
- Sampling of strain types for further studies.
- Evaluation of TB control programs (level of clustering).
- Identification of strain-specific transmission/infection rates.
- Identification of predominant strain types (clonal strains) in study populations.
- Identification of hypervirulent strains in populations.
- Investigation of the evolution of the M. tuberculosis complex.

In recent times, polymerase chain reaction (PCR) has been found to be the most sensitive technique for rapid diagnosis of M. tuberculosis. This technique, capable of amplifying minute amounts of a specific DNA sequence into millions of identical copies has revolutionalised molecular biology research.

IS6110-RFLP Analysis

The best known and investigated insertion sequence is IS6110 Differences in the copy number and locations within the genome, responsible for the high degree of IS6110 polymorphism, have predisposed this sequence to be used as a specific molecular marker for genotyping of M. tuberculosis strains [53].

The Restricted Fragment Length Polymorphism (RFLP) technique using the IS6110 repetitive sequence is considered for typing the M.tuberculosis complex strains. It involves the extraction of genomic DNA, restriction endonuclease digestion with PvoII, Southern blotting and probing for IS6110.

Nucleic Acid Hybridization Techniques

These tests used with nonisotopically labelled (acridine ester-labelled nucleic acid) probes specific for mycobacterial ribosomal RNA (rRNA). The RNA is released from cell after sonication. The DNA probe is allowed to react with the solution. If specific RNA is present, a stable DNA, RNA complex, or hybrid is formed. The complex is detected by alkaline hydrogen peroxide solution. The hybrid bound acridine ester is

available to cause a chemiluminescent reaction, resulting in the emission. The amount of light is emitted of light is related to the amount of hybridized probe [33, 34].

Direct Nucleic Acid Amplification Test

The amplified Mycobacterium direct test (AMD) consist of transcription mediated amplification of a specific 16S rRNA target at a constant temperature for detection of M. tuberculosis and NTM in smear positive and negative specimens [33, 34].

Gene Sequence Analysis

In particular, 16SrRNA, rpoB and Internal Transcribed Spacers (ITSs) have been shown to be useful for the rapid identification of many mycobacterial species [33, 34], including M. malmoense, M. szulgai, and M. flavescens, which are hardly identified with conventional methods [33, 34].

At first, analysis of gene sequences was addressed with PCR-restriction fragment length polymorphism (RFLP) analysis, otherwise called the PRA, or PCR-restriction enzyme analysis (REA), method. This method combines PCR amplification and restriction analysis. The pattern obtained after electrophoresis is species or strain specific [36].

SNP Analysis

Two major lines of research based on SNP analysis include lineage specific typing and determination of the occurrence of mutations leading to drug resistance.

The SNP at a particular location might be detected by REA [37] or by a variety of PCRs. They can be addressed with molecular beacons, as they are able to distinguish sequences that differ by even a single nucleotide substitution [38]. Next, they can be detected by identifying shifts in melting temperatures obtained by real-time PCR curve analysis. [39]

Genome Analysis PFGE

The first molecular typing methods for M. tuberculosis genome were based on RFLP analysis of bacterial DNA. Here, chromosomal DNA isolated from different mycobacterial strains is digested by using various restriction enzymes. The resulting restriction fragments are separated by gel electrophoresis and visualized with UV light. The observed fingerprint patterns are strain specific [40].

RAPD Analysis

Randomly amplified polymorphic DNA (RAPD) analysis or arbitrarily primed PCR (AP-PCR) is a typing method that has increasingly been used for estimating genetic variability among different bacterial taxon's. This method requires no previous knowledge of the template DNA sequence. By using a single, arbitrarily designed primer with a length of 5 to 50 bp and low-stringency conditions, the primer anneals to template DNA at both perfectly and partially matched sites, resulting in strain-specific multiband DNA profiles [41].

AFLP

AFLP analysis is a PCR-based method in which DNA is digested with two restriction enzymes, a rare cutter and a frequent cutter, which have 6- and 4-bp recognition sites, respectively. The resulting restriction fragments are ligated to double-strand adaptors (10 to 30 bp) recognized by PCR primers that are complementary to the adaptor sequence, carry the restriction site sequence, and contain selective bases at their 3= ends. The use of radiolabeled primers allows visualization of PCR products by means of autoradiography [42].

Deletion Mapping

Deletions or, rather, LSPs can be used as molecular markers to study genetic variability among mycobacteria. The LSP-based methodology relies on previous knowledge of the analyzed sequences, and it usually requires relatively large quantities of DNA (micrograms) [43].

Whole-genome Sequencing (WGS)

The completion of the genomic sequence of M. tuberculosis H37Rv [44] has commenced a whole new chapter in the epidemiological study of mycobacteria. The development of second-generation sequencing (SGS) and further-generation sequencing platforms has made studies on mycobacterial epidemiology as informative as they have never been before.

Spoligotyping

Clustered regularly interspaced short palindromic repeats (CRISPRs) comprise a family of widely encountered repetitive DNA elements. The

CRISPR loci generally consist of a noncoding, A/T-rich leader sequence and variable numbers of identical direct repeats (DRs) interspersed with unique spacer sequences or spacers. Adjacent to CRISPRs are often CRISPR-associated (Cas) genes, together forming a CRISPR-Cas genomic region. CRISPR loci are thought to represent a sort of prokaryotic adaptive immunity system that confers resistance to phages [47]. The number of spacers within CRISPR loci is variable. Spacers may be acquired from a viral invader as a specific way of memorizing phage infection [47]. On the other hand, some spacers may be deleted as a result of transposition and homologous recombination between neighboring or distant DRs. The mechanism of phage resistance is conferred by the expression of this sequence, hybridization, and cleavage of foreign RNA or DNA [47].

Spacer oligonucleotide typing (Spoligotyping) is a PCR-based technique for MTBC strain differentiation that takes advantage of the structure and polymorphism of the DR locus. In spoligotyping, the entire locus is amplified by PCR by using two inversely oriented primers complementary to the sequences of DRs. A biotinylated reverse primer is used so that all the reverse strands are labeled. Next, PCR products are hybridized to a membrane with a set of 43 immobilized, covalently bound, synthetic oligonucleotides, each representing a unique spacer identified by sequencing of the DR locus in M. tuberculosis H37Rv (spacers 1 to 19, 22 to 32, and 37 to 43) and M. bovis BCG vaccine strain P3 (spacers 20, 21, and 33 to 36). After hybridization, the membrane is incubated with a streptavidin-peroxidase or streptavidin-alkaline phosphatase

conjugate, and the hybridization signals are detected by chemiluminescence. Strain-specific patterns (spoligotypes) are then visualized on X-ray film. Strains are differentiated by the presence or absence of individual spacers in the complete 43-spacer set [49]. Since spoligotyping results can be presented as a binary system (present/absent), they can be easily interpreted, digitized, and compared among different laboratories [49].

Rep-PCR

This procedure involves the amplification of repetitive, noncoding sequences and their separation using microfluidic electrophoresis over a chip. As the fragments migrate over the chip, their size and fluorescence intensity are measured by a laser, thereby generating a graph.

IDENTIFICATION OF NTMS IN CLINICAL SPECIMENS

Differentiating clinical manifestation of NTMs from M. tuberculosis is very difficult. [55].

The separation and identification of NTMs requires 5-10% CO2 . On the other hand, phenotypic identification of various NTMs is dependent on at least 10 different tests; therefore, reliability of final result is usually hazy as difficulties are faced in interpretation of various tests . [57, 58].

Thus, fast and reliable diagnostic techniques for identification of NTMs need to be developed. These techniques include various molecular tests like molecular probes, hsp65 gene based algorithms, 16S rRNA sequencing techniques and non-molecular tests conjoined with key phenotypic tests [59-61].

Recent diagnostic methods

The GeneXpert

The Cepheid GeneXpert System's MTB/RIF assay is a single use cartridge-based semi-quantitative nested real-time Polymerase Chain Reaction (PCR) in-vitro diagnostic test that detects M. tuberculosis complex DNA and rifampicin resistance associated mutations of the RNA polymerase beta (rpoB) gene.

Based on a WHO meta-analysis of the sensitivity and specificity of Xpert MTB/RIF, the test has shown very high sensitivity in sputum samples, 98% in smear-positive, culture positive and 79% in people living with HIV. Regarding extrapulmonary samples, the test shows high sensitivity when compared to culture, in diagnosing extrapulmonary TB from lymph node tissues or aspirates (84.9%), gastric lavage (83.8%), cerebrospinal fluid (79.5%) and other tissue specimens (81.2%). By contrast pleural fluid samples did not demonstrate good sensitivity (43.7%). The specificity is notably high in all groups, more than 92.5%.

DRUG SUSCEPTIBILITY TESTS FOR THE ASSESSMENT OF DRUG SENSITIVITY AND RESISTANCE

Nowadays, despite of passing more than 70 years of discovery of the first anti-tuberculosis antibiotic [streptomycin in 1943] , tuberculosis has been a serious issue and one the most important cause of mortality yet.

The known reasons of crescent resistance of mycobacterium tuberculosis to drugs can be: not applying the appropriate remedy (improper drug prescription, using one-drug instead of multi-drug remedies, interruption in drug appliance), lack of appropriate control of tuberculosis, decrease in effectiveness of anti-tuberculosis drugs and finally prevalence of HIV [64].

The treatment of infections caused by TB and NTM is generally obtained by surgery, drug appliance or both of them. Drug treatment of these diseases is quite costly, protracting and very often followed by consequences of drug toxicity. In case of resistant strains of mycobacterium tuberculosis, first and second generations of therapeutic drugs are not effective. The suggested remedy for most of the slow-growing species consists of rifampin, Ethambutol and macrolide antibiotics for 18-24 months; in more complicated cases the necessity of drug susceptibility tests is mandatory. For fast-growing species the remedy is often chosen according to drug susceptibility tests. In rarer cases (like M. abscessus) steady therapeutic drugs consist of macrolide amikacin and tigecycline are preferred [65]. Different methods to determine the microbial susceptibility of mycobacterium species are :

Absolute Concentration Method

In this method, the species of interest should be standardly inoculated in mediums of different drug concentrations. One of these mentioned concentrations is the critical concentration determining the Minimum Inhibitory Concentration (MIC). The growth in critical and higher

concentrations indicates the resistance of the mentioned species and is compared with drug free mediums with the intention of controlling [66, 67].

Inhibition Ratio Method

This method is similar to absolute concentration method but the MIC is determined according to standard strain H37Rv of mycobacterium. Subsequently, the result is the ratio of drug MIC which doesn't inhibit the growth to MIC needed to inhibit the growth of the standard strain H37Rv. This method is more commonly used for M. tuberculosis strains. [66, 67].

Proportion Method

This method which is still suggested by WHO for testing the second-line therapy drugs includes the usage of drug containing mediums that two prepared standard concentrations of bacteria are sequentially fecundated in mediums containing the drug and not containing it. The number of colonies formed in drug containing mediums is calculated according to the most dilute inoculation and then is compared to the number of colonies formed in drug free mediums of the same concentration. If the ratio of bacilli grown on the drug containing medium to the non-containing ones is more than 1%, the strain would be resistant to the used drug [66, 67].This method is not applicable for pyrazinamide.

Disk Diffusion Method

In this method a disk with a certain amount of anti-microbes is placed on the solid medium which the examined bacteria is cultured on it. The zone of inhibition around the disk is compared with the scales

determined by CLSI. This technique is chiefly used to determine the microbial susceptibility of fast-growing species.(67).

Disk Elution Method

In this method, the disks containing a certain amount of antibiotic are added to liquid Oleic Albumin Dextrose Catalase (OADC) growth supplement and then are added to the melted medium. Mix the contents and then divide them among the plates of growth medium at room temperature in order that a solid state is obtained. The results of growing or not growing are reported according to the amount of antibiotic that each disk contains [68].

Broth Micro-Dilution Method

Broth micro-dilution has been stated as the golden standard with the intention of drug resistance test in bacteriology. It is performed in 96-well plates which a series of antibiotic concentrations is inserted in them. Then every well is filled with 100 uL of a suspension formed of the bacteria of interest in a broth medium with a concentration about 5-10*5. The control well is only filled with bacteria suspension. In this experiment, the MIC is defined as the lowest concentration of antibiotic which bacteria does not grow in it [72, 73].

Epsilon Tests

This test was used for the first time in 1990 in order to determine the Mycobacterial antibiotic resistant. Determining the MIC using this method begins with the appliance of plastic strips impregnated with

decreasing antibiotic concentrations. Numbering the MIC is similar to proportion method or certain concentration method. Using two antibiotic for one strain at the same time is one the advantages of this method [74].

Role of the Health Department in Infection Control-RNTCP

Health department TB control programs should not only ensure that each of their clinics develop a TB infection-control program; they should also be able to provide consultation about TB infection control to other health care and congregate settings in their communities.

Specifically, health departments should be able to assist health care settings with:

- Understanding the importance of infection control principles
- Reporting confirmed or suspected TB cases as quickly as possible
- Conducting contact investigations
- Ensuring there is a plan for TB patients to receive follow-up care after they are discharged
- Conducting risk assessments, testing, surveillance, outbreak investigations, and other aspects of a TB infection-control program
- Planning and implementation of TB control activities.

TB Infection- Goals of Control Program

The main goals of a TB infection-control program are to ensure early and prompt

- Detection of TB disease
- Airborne precautions (e.g., isolation of people with known or suspected TB disease)

- Treatment of people with known or suspected TB disease.

The main goals of an infection-control program are to detect TB disease early and to promptly isolate and treat people who have TB disease.

MATERIALS & METHODS

MATERIALS AND METHODS

This prospective study was conducted in Coimbatore Medical College for a period of 12 months from June 2017 to May 2018. Sixty four patients with suspected tuberculous lymphadenitis attending outpatient department of medicine and surgery , Coimbatore Medical College Hospital, Coimbatore were included in the study.

Sample Collection

Fine Needle Aspirate;

Fine needle aspirates were obtained by the pathologist after following adequate sterile precautions and the specimen were processed in the Diagnostic Laboratory, Microbiology , and cytology was reported by the pathologist in Coimbatore Medical College.

After getting consent from patients , the aspiration was collected with sterile precautions ,the sample was smeared on four slides and three were fixed in 100% isopropyl alcohol for cytology by Haematoxylin and Eosin method and the fourth slide was evaluated for AFB by Ziehl Neelsen method. One other portion of the material was collected in an eppendorf centrifuge tube and stored at -80ºC for PCR.

Laboratory Safety

M.tuberculosis has a very low infective dose as exposure to fewer than 10 acid fast bacilli leads to 50% infection rate. All the safety measures were taken during sample collection, used leak proof unbreakable containers. 5% phenol was used as disinfectant solution. Biosafety level-2

practices and procedures are required for preparation of acid-fast smears (non aerosol). All aerosol generating activities must be conducted in a Biosafety cabinet. This offers adequate protection to the worker against inhalation of aerosols.

Cleaning of the laboratory was done as per biomedical guidelines with clear phenolic disinfectants and solutions of sodium hypochlorite and the room was periodically fumigated with formaldehyde.

Cytology

Fixation prevents the post mortem changes and cells are preserved as close as possible to living state so as to make a proper cytologic study. Fixation was done in this study by using 100% isopropyl alcohol for 20 minutes.

Interpretation of cytology: It was done as follows by the pathologist.

The cytomorphological criteria considered for diagnosis;

1) FNA cytology showing epitheloid cell granulomas with or without multinucleated giant cells and caseation necrosis.
2) In case of cytology showing necrosis only/non-caseating granulomas / acute suppurative lymphadenitis without demonstration of AFB were considered as suspicious of tuberculosis.

Examination of Ziehl-Neelsen Stained Smear of the Fine Needle Aspirate

Preparation of Smear

Marked the specimen number on the clean glass slide with a diamond pencil.

Used one slide for each specimen. With a 5mm internal diameter 24 SWG Nichrome wire loop the specimen was smeared in an uniform layer on about two thirds of the slide.

Water Quality is Key

AFB microscopy is not specific – As acid fast environmental contaminants as well as NTM and MTB presence in the specimen will be detected. Introduction of an environmental contaminant during wash steps and in reagent preparation must be avoided. Use of a negative control slide essential for detecting potential environmental contaminants.

Quality Assurance of AFB Microscopy

A known positive and known negative smear should be read with each run and when a new reagent is used. QC smears may be prepared in advance, heat-killed, and stored unstained. Preferable to prepare QC slides using sediment from a clinical specimen. Patient smears should only be examined and reported when control slides are acceptable.

Smear fixation : Smear are fixed by flaming before staining

Ziehl-Neelsen Staining Procedure

1) Placed the slide on the staining rack with the smeared side up.
2) Flooded entire slide with strong carbol fuchsin which is fitered through Whatman No.1 Paper.

3) Heated the slide slowly until it was steaming. Avoid boiling. Maintained steaming for 5 min by using intermittent heating. Should not allow the stain to dry on the slide.

4) The slide is rinsed in a gentle stream of running water until all free stain is washed away.

5) Flooded the slide with 20% sulphuric acid as a decolourising agent for 2 mins.

6) Rinsed the slide thoroughly with water. Drained excess water from the slide.

7) Flooded the slides with 0.1% methylene blue counter stain for one minute.

8) Rinsed the slide thoroughly with water

9) Allowed the smear to air dry.

10) Viewed under oil immersion Microscope.

MOLECULAR TECHNIQUE

POLYMERASE CHAIN REACTION

Real-time PCR Detection MTB-IS6110

PureFast® Bacterial DNA mini spin purification kit [Kit contains Proteinase-K, Lysis buffer, Wash Buffer-1, Wash Buffer-2, Spin columns with collection tube and elution buffer. HELINI MTB Real-time PCR kit is from HELINI Biomolecules, Chennai, India.

Real-time PCR Model: Agilent AriaMx, USA

- HELINI MTB Real-time PCR kit components:

- Probe PCR Master Mix
- MTB Primer Probe Mix
- Internal control Primer Probe Mix
- Internal control template
- Positive control
- Instruction manual

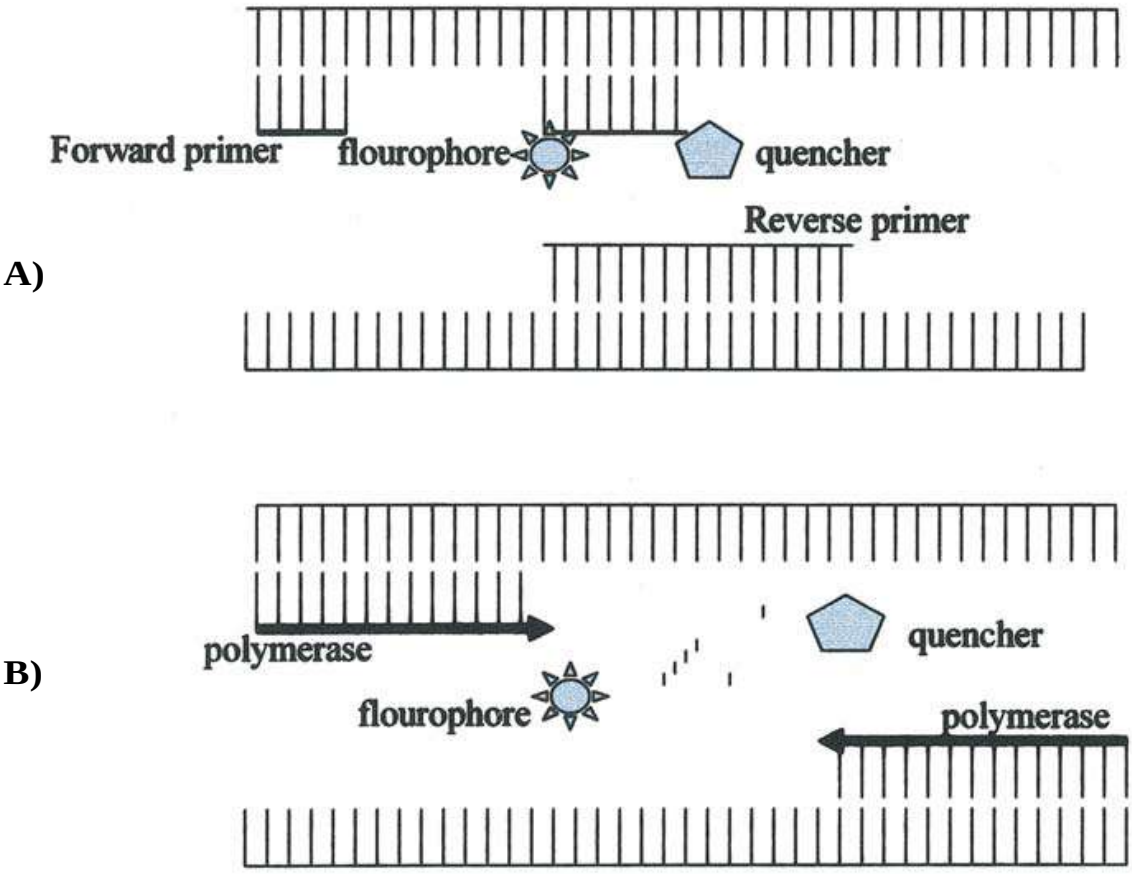
DNA Purification

1. 100µl of lym node sample transferred into fresh 1.5ml centrifuge tube. 100µl sterile water added and mixed well.
2. 200µl Bacterial Lysis buffer added. Mixed well by vortex
3. Add 20µl of Proteinase K and 5µl of internal control template, Mixed well by inverting several times.
4. Incubate at 56ºC for 15min.
5. Added 220µl of Ethanol and mixed well.
6. Transferred entire sample into the spin column. Centrifuged at 10000rpm for 1 min. Discard the flow-through and place the column back into the same collection tube.
7. Added 500µl Wash buffer-1 to the spin column. Centrifuge at 10000rpm for 1min and discard the flow-through. Place the column back into the same collection tube.
8. Added 500µl Wash buffer-2 to the spin column. Centrifuge a 10000rpm for 1min and discard the flow-through. Place the column back into the same collection tube.

9. Discard the flow-through and centrifuge for an additional 1 min. This step is essential to avoid residual ethanol.
10. Transferred the spin column into a fresh 1.5 ml micro-centrifuge tube.
11. Added 60µl of Elution Buffer to the center of spin column membrane.
12. Incubate for 1 min at room temperature and centrifuge for 2 min.
13. Discard the column and store the purified DNA at -20°C. 10µl of elute used in real-time PCR analysis.

PRINCIPLE

Taq Man probes: Taq Man probes are oligonucleotides that contain a fluorescent dye, typically on the 5' base and a quenching dye typically located on the 3' base. When irradiated, the excited fluorescent dye transfers energy to the nearby quenching dye molecule rather than fluorescing, resulting in a nonfluorescent substrate. Taq Man probes are designed to hybridize to an internal region of a PCR product. During PCR when the polymerase replicates a template on which a Taq Man probe is bound, the 5' exonuclease activity of the polymerase cleaves the probe4. This separates the fluorescent and quenching dyes and FRET no longer occurs. Fluorescence increases in each cycle, proportional to the rate of probe cleavage.



Overview of TaqMan format: Steps - (A) TaqMan probe contains fluorophore at the 5' end and the quencher at 3' end. The proximity between quencher and fluorophore permits FRET resulting in a nonfluorescent substrate. (B) By the 5'-exonuclease activity of the Taq polymerase the probe is hydrolyzed and reporter dye is separated from the quencher, resulting in an increase in fluorescence emission.

qPCR Procedure:

Detection Mix

Components	**HBV**
Probe PCR Master Mix	10µl
MTB Primer Probe Mix	2.5µl
Internal control Primer Probe Mix [IC PP Mix]	2.5µl
Purified DNA	10µl
Total reaction volume	25µl

Centrifuged PCR vials briefly before placing into thermal cycler.

Negative Control

Included 10µl of nuclease free water

Positive Control

Included 10µl of Positive control

Amplification Protocol

	Step	**Time**	**Temp**
	Taq enzyme activation	15min	95ºC
45 cycles	Denaturation	20sec	95ºC
	Annealing/Data collection*	20sec	56ºC
	Extension	20sec	72ºC

MTB = FAM channel

Internal Control = HEX Channel

Interpretation of RT-PCR

- Following run evaluation
    - Valid positive and negative control
    - Specimen has a normal curve
- Record the cycle threshold (Ct) values
    - If a sample has no cycle threshold values (0.00) it is negative
- Determine if there are any suspect samples
    - Weak positives-Ct values >35.

GENEXPERT MTB/RIF

PROCEDURES

General and safety procedures

- All processing of clinical specimens should be performed as per the biosafety standards.
- Treat all sputum specimens, including used cartridges, as potentially infectious.
- Wear protective gloves and laboratory coats when handling specimens and reagents.
- Wash hands thoroughly after handling specimens and test reagents.
- All benches should be decontaminated after work, or immediately after a spill, with an appropriate mycobactericidal disinfectant followed by 70% alcohol.

Equipment & Materials

- GeneXpert Dx system equipped with GX2.1 software/computer/printer/barcode wand-reader and operator manual (Cepheid Inc, Sunnyvale, USA) (Cat Item No GXIV-4N1-6).
- Class II biological safety cabinet (BSC)
- Xpert MTB/RIF kit- contains 10 individually packed cartridges:
    - Store at 2-28°C.
- The sample reagent solution (8ml/catridge) is clear, but may range from colorless to golden yellow.
- Permanent marker pens.
- Sterile (individually packed) disposable transfer pipettes.
- Sterile screw-capped specimen collection containers/cups.

Specimen Processing

1. As lymph node aspirate is homogeneous, it is mixed with sterile phosphate buffer in the ratio 1:2.
2. Vigorous shaking for 10 to 20 times or vortex for atleast 10 seconds.
3. The mixture is incubated at room temperature for 10 minutes.
4. Then again shake the specimen vigorously for 10 to 20 times or vortex for atleast 10 seconds and incubate for 5 minutes.
5. Now 2 ml of processed sample is taken and loaded in the cartridge.

Preparing the Cartridge

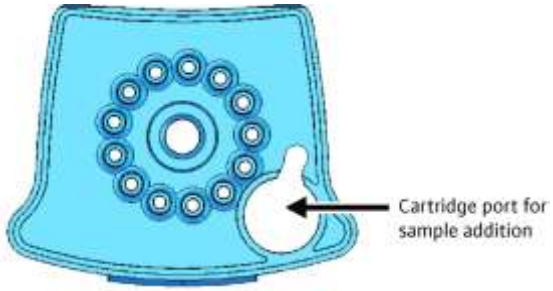


Figure 1: Xpert MTB/RIF cartridge (top view)

- Carefully put the pipette back into the paper/plastic cover. Discard the transfer pipette into bio-hazard waste bin.
- Close the cartridge lid. Make sure the lid snaps firmly into place and load the cartridge into the GeneXpert Dx instrument.
- Start the test within 30 minutes of preparing the cartridge.
- The minimum required amount to be loaded into the cartridge is 2ml (as marked on the Pasteur pipette).
- Remaining liquefied sample may be kept for up to 12 hours at 2-8°C (for repeat testing).
- Uninterrupted power source (UPS).
- In the Sample ID box, type the Laboratory number must match the number on the cartridge and on sputum cup.
- Run the test. Test takes around 1 hour 55 minutes to complete run.
- The result print-out is generated automatically.
- Dispose the used cartridge in the biohazard waste container.

Quality controls

Each Xpert MTB/RIF cartridge includes a Sample processing control (SPC) and Probe Check control (PCC). Print out of the test result indicates the validation of controls.

1) Sample Processing Control (SPC): Ensures the sample was correctly processed. The SPC contains non-infectious spores in the form of a dry spore cake that is included in each cartridge to verify adequate processing of MTB. SPC should be positive in a negative sample and can be negative or positive in a positive sample. The SPC passes if it meets the validated acceptance criteria. The test result will be “Invalid” if the SPC is not detected in a negative test.
2) Probe Check Control (PCC): Before the start of the PCR reaction, the GeneXpert Dx system measures the fluorescence signal from the probes to monitor bead hydration, reaction-tube filling, probe integrity and dye stability. Probe Check passes if it meets the assigned acceptance criteria.

ERROR

- MTB-NO RESULT
- SPC-NO RESULT
- Probe Check-NA (not applicable)

Interpretation of Results

- This is DNA based test, meant for New TB suspect, ensure don't enrol 'follow-up' patient. The results are interpreted by the GeneXpert Dx system from measured fluorescent signals and embedded calculation algorithms and will be displayed in the "View Results" window. Lower Ct values represent a higher starting concentration of DNA template; higher Ct values represent a lower concentration of DNA template.

MTB DETECTED

- If MTB target DNA is detected- the MTB result will be displayed at High, Medium, Low or Very Low depending on the Ct value of the MTB target present in the sputum sample. Below table lists the Ct value ranges for the displayed MTB results:

MTB result	Ct range
High	<16
Medium	16-22
Low	22-28
Very Low	>28

- Rifampicin resistance result types, when MTB is detected:
    - Rifampicin resistance DETECTED: a mutation in the rpoB gene has been detected that falls within the valid delta Ct setting.

- Rifampicin resistance NOT DETECTED: no mutation in the rpoB has been detected.
- Rifampicin resistance INDETERMINATE: the MTB concentration was very low and resistance could not be detected.

Limitations of this In vitro diagnostic test (IVD)

- Reliable results depend on proper specimen collection, handling, and storage.
- A positive test result does not necessarily indicate the presence of viable organisms. It is however, presumptive for the presence of MTB and rifampicin resistance.
- The results might be affected by antecedent or concurrent anti-TB drug therapy.

FIG 1: AFB SMEAR ZIEHL NEELSEN METHOD

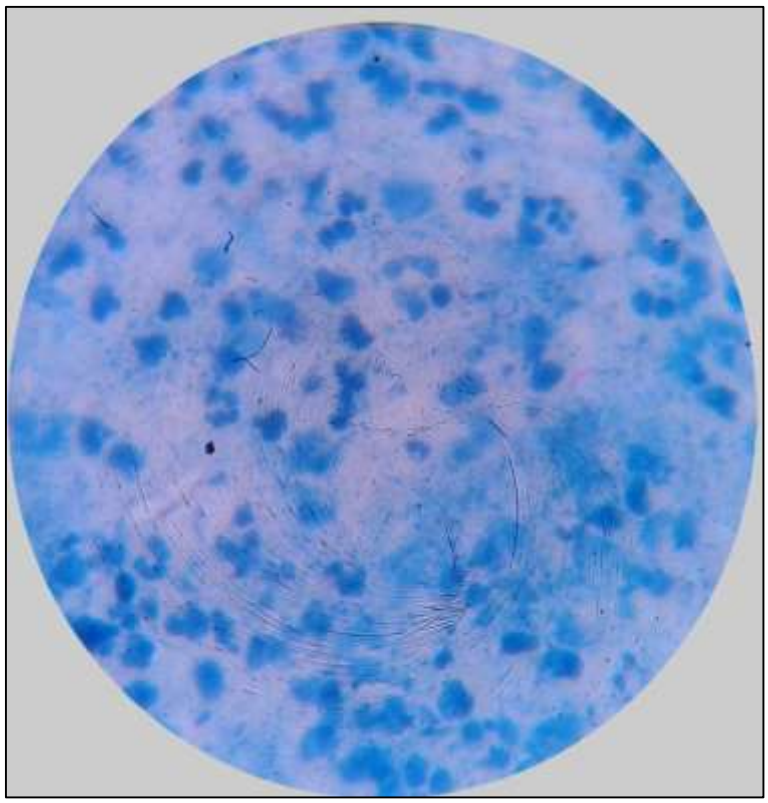


FIG 2: CYTOLOGY - HEMATOXYLIN AND EOSIN STAIN

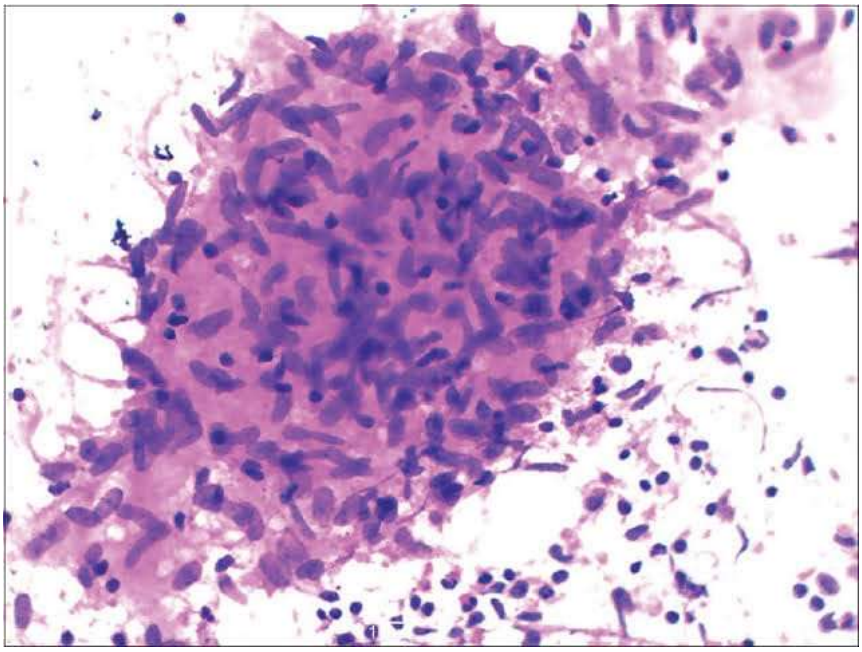
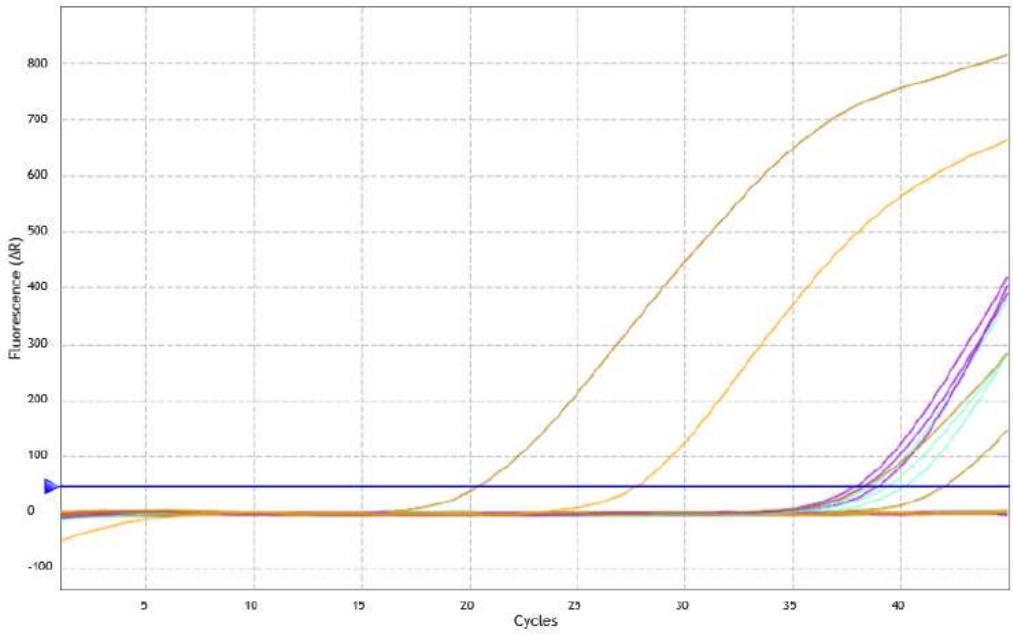


FIG 3: RT-PCR

Amplification Plots



Threshold settings based on background: Start cycle = 1, End cycle = 1, Sigma multiplier = 10, Baseline correction = Adaptive, Smoothing = On, Crosstalk Correction: -

Tabular Results

Well	Well Name	Dye	Cq (ΔR)
A2	NTC	FAM	No Cq
A4	8	FAM	38.72
A6	16	FAM	No Cq
A8	PC	FAM	20.53
B2	1	FAM	No Cq
B4	9	FAM	No Cq
B6	17	FAM	38.40
C2	2	FAM	42.11
C4	10	FAM	No Cq
C6	18	FAM	No Cq
D2	3	FAM	No Cq
D4	11	FAM	No Cq
D6	19	FAM	No Cq
E2	4	FAM	40.23
E4	12	FAM	38.98
E6	20	FAM	No Cq
F2	5	FAM	No Cq
F4	13	FAM	No Cq
F6	21	FAM	No Cq
G2	6	FAM	No Cq
G4	14	FAM	No Cq
G6	22	FAM	39.46
H2	7	FAM	37.97
H4	15	FAM	38.48

Well	Well Name	Dye	Cq (ΔR)
H6	23	FAM	27.82

FIG 4: GeneXpert IV SYSTEM

FIG 5: GeneXpert CARTRIDGE

RESULTS

RESULTS

A total of 64 clinically suspected patients of tuberculous lymphadenitis are included in the present study. The period lasted for one year from June 2017 to May 2018.

Out of 64 patients, 41 patients presented only with cervical lymphadenopathy of variable duration. People with swelling in the neck , loss of weight and fever accounted for 25% . Remaining 10% of the study group presented with swelling in the neck and fever.

The following results are obtained and tabulated;

TABLE 1: AGE-WISE DISTRIBUTION OF THE STUDY PARTICIPANTS

Age Group	No. of Cases
15-30 years	36 (56.25%)
31-45 years	16 (25%)
45-60 years	9 (14%)
More than 60 Years	3 (4.75%)

Out of 64 cases , 36 (56.25%) patients were between 15-30 years, 16(25%) patients were between 31 – 45 years, 9 (14%) were between 45 – 60 years and 3 (4.6%) were over 60 years.

Maximum cases were recorded in the age group between 15-30 years.

TABLE 2: GENDER-WISE DISTRIBUTION OF THE STUDY PARTICIPANTS

Sex category	No. of Cases
Female	38 (59%)
Male	26 (41%)
Total	**64 (100.0%)**

Out of 64 samples, 38 (59%) patients were female and 26 patients were males (41%) showing female preponderance.

TABLE 3: COMPARISON OF AGE & GENDER OF THE STUDY PARTICIPANTS

	Males	Females
15-30 years	16	20
31-45 years	6	10
45-60 years	3	6
More than 60 years	1	2
Total	**26**	**38**

In this table, females preponderance is seen in all age group.

TABLE 4: TYPES OF SAMPLE BY FNA

Types of sample	No. of Cases
Aspirate	55
Pus	9
Total	**64**

Out of 64 samples, 55 (86%) samples were aspirate and 9 (14%) were pus.

TABLE 5: SITES OF ASPIRATION

Site of Aspiration	Right Side (n=43)	Left Side (n=21)
Posterior Triangle	21	16
Anterior Triangle	11	3
Submandibular	1	0
Supraclavicular	10	2

Out of 64 samples , 37 (57.8%) samples were from posterior triangle, 14 (21.8%) samples from anterior triangle and 12 (18.75%) samples from supraclavicular nodes and 1 (1.5%) from submandibular nodes.

TABLE 6: CLINICAL OBSERVATION

Clinical History	No. of Cases
Swelling only	41 (64%)
Swelling & Loss of weight & Fever	16 (25%)
Swelling & Fever	7 (11%)

16 (25%) patients showed typical history whereas remaining 48(75%) patients presented without constitutional symptoms.

TABLE 7: IMMUNOLOGICAL STATUS

Immunological Status	No. of Cases
Immunosuppressant	6 (9%)
Immunocompetant	58 (91%)
Total	**64 (100%)**

Out of 64 samples, 6 (9%) samples from immunocompromised patients and remaining 58 samples (91%) were without comorbid condition.

TABLE 8: CYTOLOGY-HAEMATOXYLIN AND EOSIN STAINING

Cytology	No. of Cases
Positive	22 (34%)
Negative	42 (66%)
Total	**64 (100%)**

Out of 64 samples, 22 (34%) samples showed cytomorphological features of tuberculous lymphadenitis and 42 (66%) samples were negative.

TABLE 9: NATURE OF CYTOMORPHOLOGY

Nature of smears	AFB			P value
	Positive	Negative	Total	
Necrosis with Neutrophils	10	7	17	**<0.004**
Epitheloid cell granulomas with caseous necrosis	7	15	22	0.78
Epitheloid cell granulomas without caseous necrosis	2	12	14	0.15
Necrosis +/- lymphocytes	0	3	3	0.24
Reactive lymphoid cells with nonspecific inflammation	0	8	8	**0.049**
Total	19	45		

Out of 64 samples ,positive cytomorphological features obtained in 22 samples whereas suspicious of TB accounts for 34 samples and remaining 8 samples were reactive lymphadenitis.

TABLE 10: AFB SMEAR – ZIEHL NEELSEN TECHNIQUE

AFB	No. of Cases
Positive	19 (30%)
Negative	45 (70%)
Total	**64 (100%)**

Out of 64 samples, 19 (30%) samples were positive and 45 (70%) samples were negative.

TABLE 11: MOLECULAR METHODS - BY PCR

PCR	No. of Cases
Positive	9 (39%)
Negative	14 (61%)
Total	**23 (100%)**

Out of 23 samples, 9 (39%) samples were positive and 14 (61%) were negative.

TABLE 12: MOLECULAR METHODS - ii) GENE XPERT

Gene Xpert	No. of cases
Positive	11 (55%)
Negative	9 (45%)
Total	**20 (100%)**

Out of 20 samples 11(55%) samples were positive and 9 (45%) were negative.

TABLE 13: COMPARISON OF DIFFERENT NON-CULTURE METHODS WITH PCR

	Cytology	AFB	PCR
Positive	7 (30.4%)	9 (39.1%)	9 (39.1%)
Negative	16 (69.6%)	14 (60.9%)	14 (60.9%)
Total	**23 (100%)**	**23 (100%)**	**23 (100%)**

Out of 23 samples, cytology showed 7 positivity ,AFB smear and PCR showed 9 positivity.

TABLE 14: COMPARISON OF DIFFERENT NON-CULTURE METHODS WITH GENE XPERT

Comparison of different non culture Methods	**Cytology**	**AFB**	**Gene Xpert**
Positive	6 (30%)	4 (20%)	11 (55%)
Negative	14 (70%)	16 (80%)	9 (45%)
Total	**20 (100%)**	**20 (100%)**	**20 (100%)**

Out of 20 samples ,Cytology showed 6 positives ,AFB showed 4 positives, Gene Xpert showed 11 positives.

TABLE 15: COMPARISON OF CYTOLOGY WITH AFB

		CYTOLOGY		
		Positive	**Negative**	**Total**
AFB	**Positive**	7	12	**19**
	Negative	15	30	**45**
	Total	**22**	**42**	**64**

Out of 19 AFB positive cases ,Cytology showed 12 negatives. Out of 22 Cytology positive cases, AFB showed 15 negatives.

TABLE 16: CONSIDERATION OF CYTOLOGY WITH AFB STAINING METHOD

Consideration of Cytology method with AFB staining		
S.No	**Parameters**	**Values**
1	Sensitivity	36.84%
2	Specificity	66.66%
3	Positive Predictive Value	31.81%
4	Negative Predictive Value	71.42%

Considering with AFB smear ,cytology showed specificity 66.66% and negative predictive value is 71.42%

TABLE 17: COMPARISON OF CYTOLOGY WITH PCR

		PCR		
		Positive	**Negative**	**Total**
CYTOLOGY	**Positive**	3	4	**7**
	Negative	6	10	**16**
	Total	**9**	**14**	**23**

Out of 9 PCR positives , Cytology showed 4 negatives and out of 7 Cytology positives, PCR showed 4 negatives.

TABLE 17: CONSIDERATION OF CYTOLOGY WITH PCR METHOD

Consideration of Cytology with PCR method		
S.No	**Parameters**	**Values**
1	Sensitivity	33.3%
2	Specificity	71.43%
3	Positive Predictive Value	42.85%
4	Negative Predictive Value	62.5%

Considering with PCR method , cytology showed 71.43% specificity and negative predictive value is 62.5%.

TABLE 18: COMPARISON OF AFB WITH PCR

		PCR		
		Positive	**Negative**	**Total**
AFB	**Positive**	6	3	**9**
	Negative	3	11	**14**
	Total	**9**	**14**	**20**

Out of 9 PCR positives, AFB showed 3 negatives and out of 9 AFB positives PCR showed 3 negatives.

TABLE 19: CONSIDERATION OF AFB WITH PCR METHOD

Consideration of AFB with PCR method		
S.No	**Parameters**	**Values**
1	Sensitivity	66.67%
2	Specificity	78.57%
3	Positive Predictive Value	66.67%
4	Negative Predictive Value	78.57%

Considering with PCR method ,AFB smear showed 66.67% sensitivity and 78.57% specificity.

TABLE 20: COMPARISON OF CYTOLOGY WITH GENE XPERT

		Gene Xpert		
		Positive	**Negative**	**Total**
CYTOLOGY	**Positive**	2	4	**6**
	Negative	9	5	**14**
	Total	**11**	**9**	**20**

Out of 11 Gene Xpert positives , Cytology showed6 2 positives and out of 6 Cytology positive , Gene Xpert showed 4 negatives.

TABLE 21: CONSIDERATION OF CYTOLOGY WITH GENE XPERT METHOD

S.No	Parameters	Values
1	Sensitivity	18.18%
2	Specificity	55.55%
3	Positive Predictive Value	33.33%
4	Negative Predictive Value	35.71%

Considering with Gene Xpert , Cytology showed specificity of 55.55% and negative predictive value of 35.71%.

TABLE 22: COMPARISON OF AFB WITH GENE XPERT

		Gene Xpert		
		Positive	**Negative**	**Total**
AFB	**Positive**	4	0	**4**
	Negative	7	9	**16**
	Total	**11**	**9**	**20**

Out of 11 Gene Xpert positive , AFB showed 4 positive and out of 4 AFB positive GeneXpert showed all the 4 positive.

TABLE 23: CONSIDERATION OF AFB WITH GENE XPERT METHOD

S.No	Parameters	Values
1	Sensitivity	36.36%
2	Specificity	100%
3	Positive Predictive Value	100%
4	Negative Predictive Value	56.25%

Considering with Gene Xpert , AFB showed 100% specificity and 100% positive predictive value.

Statistical Analysis :

	TP	TN	FP	FN	SE	SP	PPV	NPV	LRP	LRN	DOR	DAE	YI	CHI2	P
PCR Vs FNAC	3	10	4	6	0.33	**0.71**	0.43	0.63	1.17	0.93	1.25	0.57	**0.05**	0.06	0.81
PCR Vs AFB	6	11	3	3	0.67	**0.79**	**0.67**	**0.79**	3.11	**0.42**	7.33	**0.74**	0.45	4.70	**0.03**
GeneX Vs FNAC	2	5	4	9	**0.18**	0.56	0.33	0.36	**0.41**	1.47	0.28	0.35	**-0.26**	1.62	0.20
GeneX Vs AFB	4	9	0	7	0.36	1.00	1.00	0.56	NA	0.64	NA	**0.65**	0.36	4.09	**0.04**
AFB Vs FNAC	7	30	15	12	0.37	0.67	0.32	0.71	1.11	0.95	1.17	0.58	**0.04**	0.07	0.79

CHART 1: AGE-WISE DISTRIBUTION OF THE STUDY PARTICIPANTS

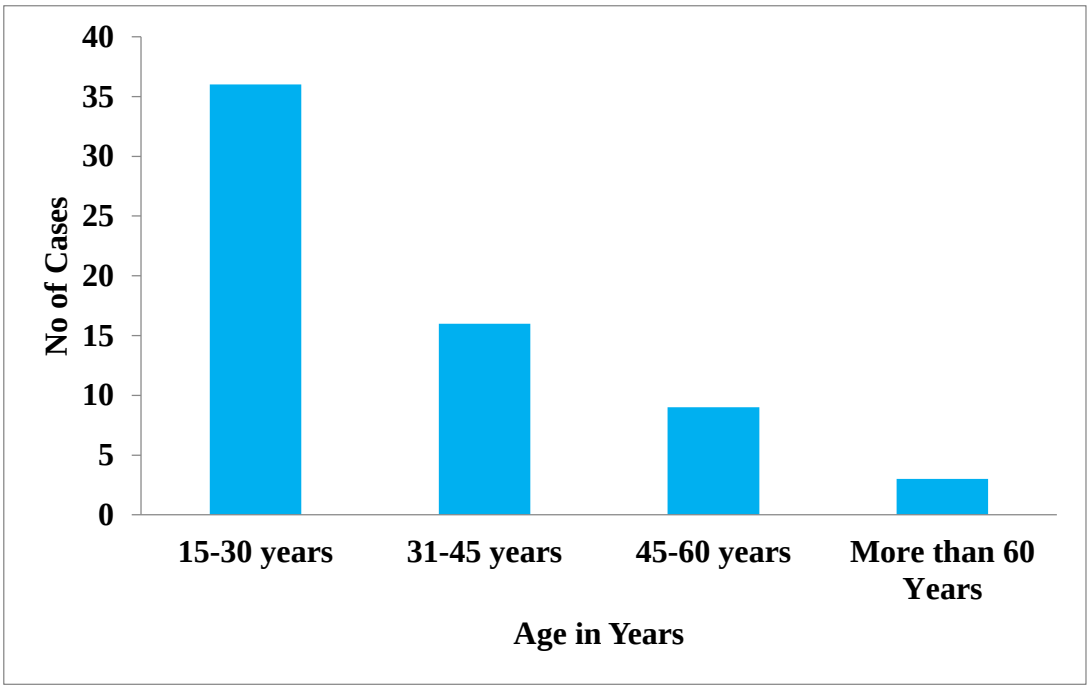


CHART 2: GENDER-WISE DISTRIBUTION OF THE STUDY PARTICIPANTS

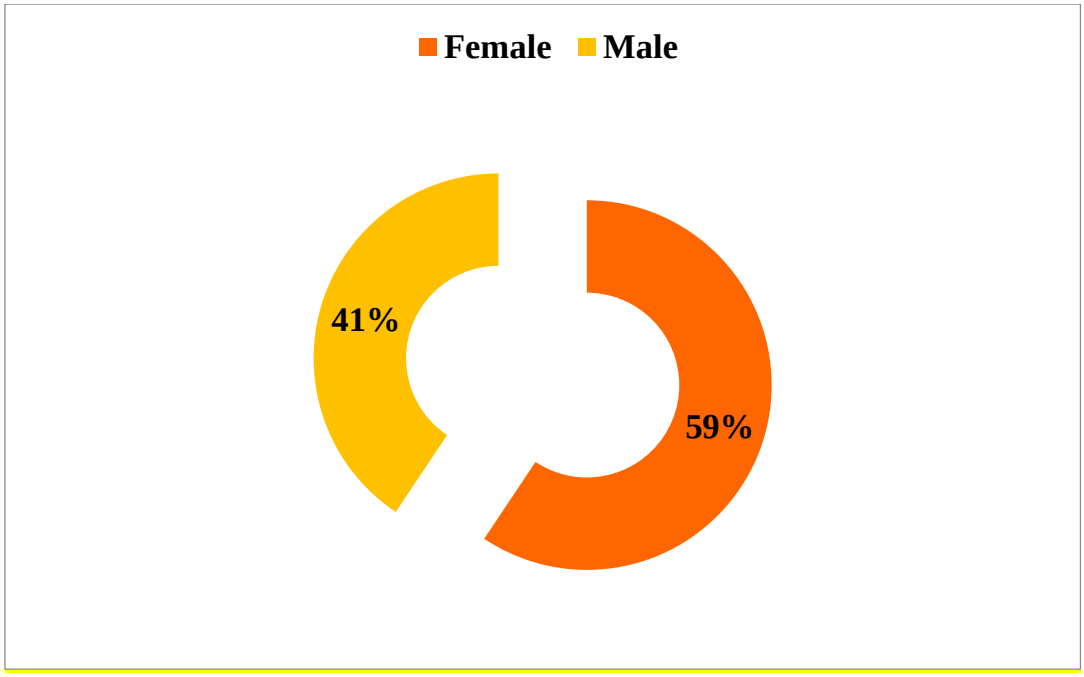


CHART 3: COMPARISON OF AGE & GENDER OF THE STUDY PARTICIPANTS

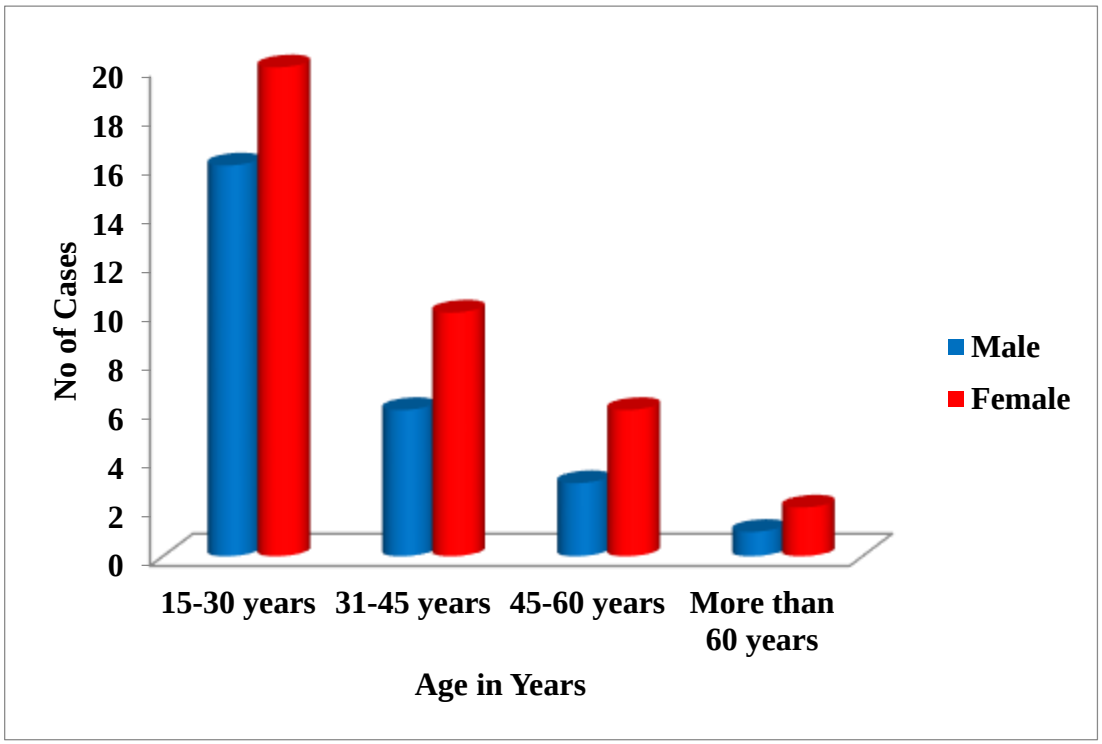


CHART 4: TYPES OF SAMPLE OF FNA

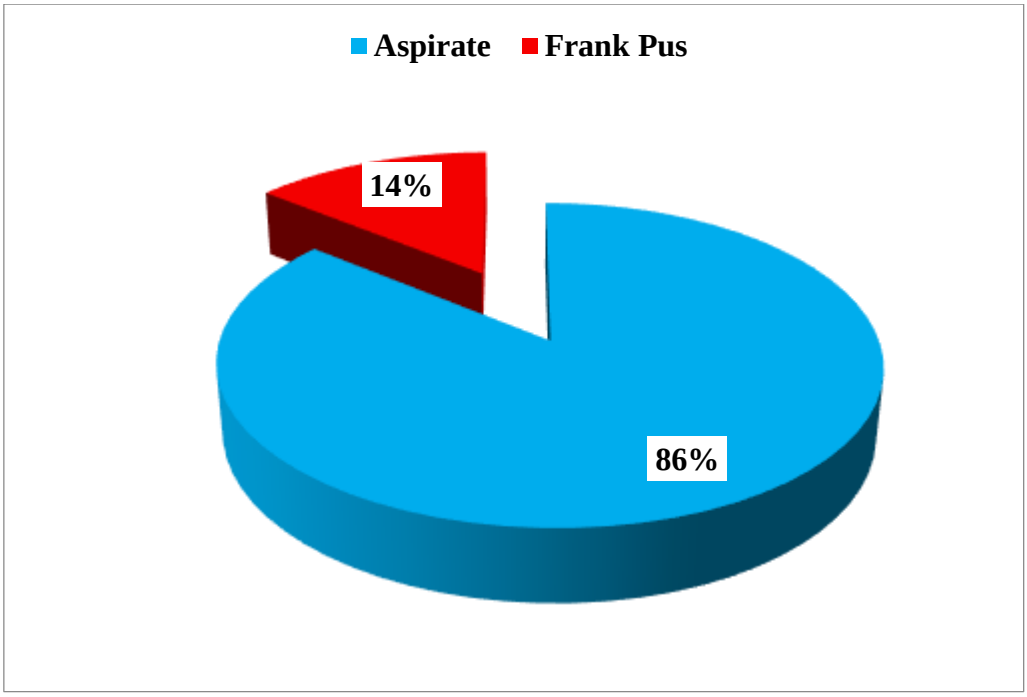


CHART 5: SITES OF ASPIRATION

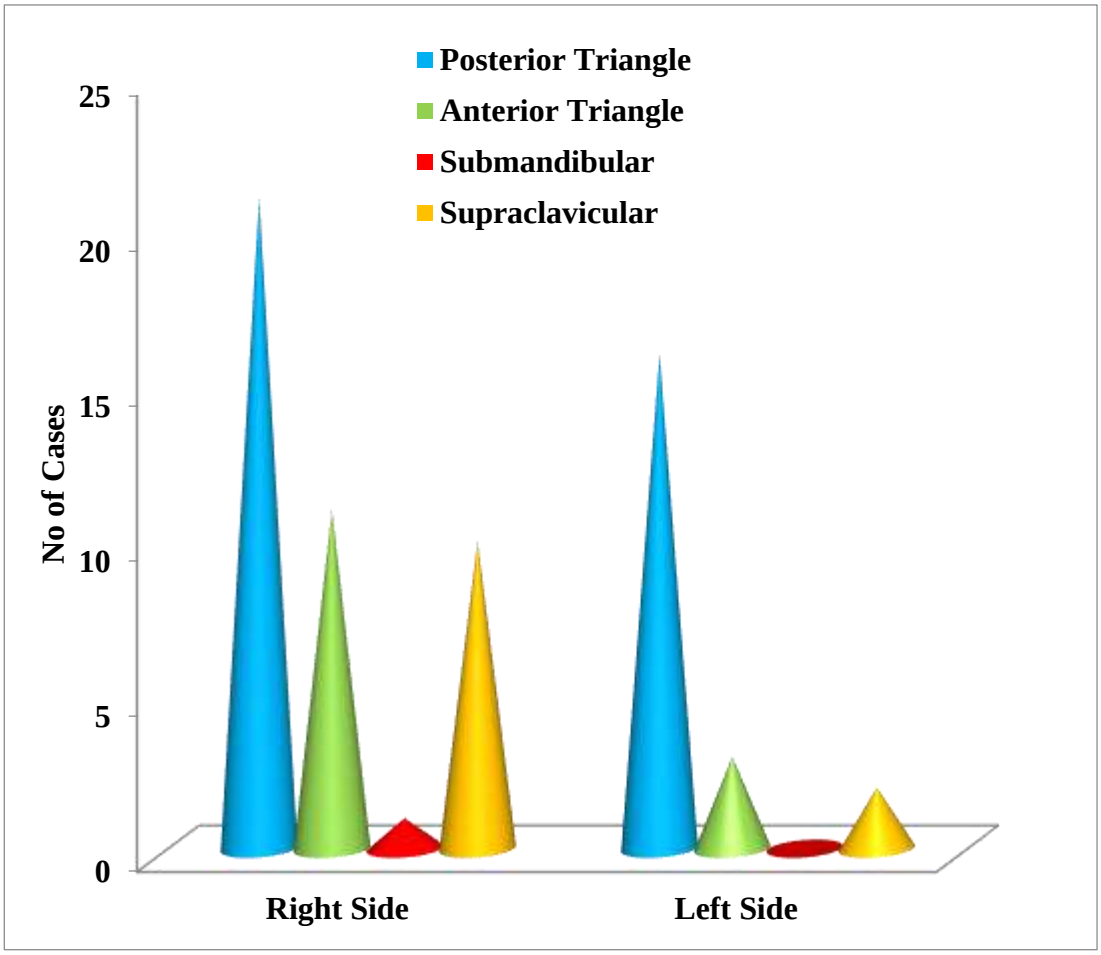


CHART 6: CLINICAL OBSERVATION

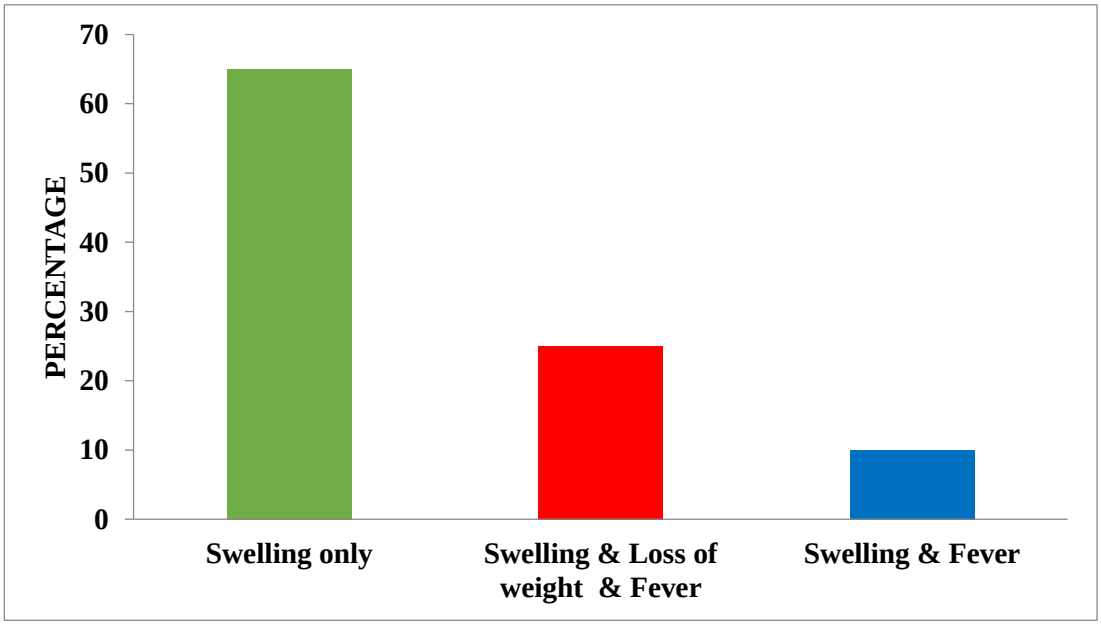


CHART 7: IMMUNOLOGICAL STATUS

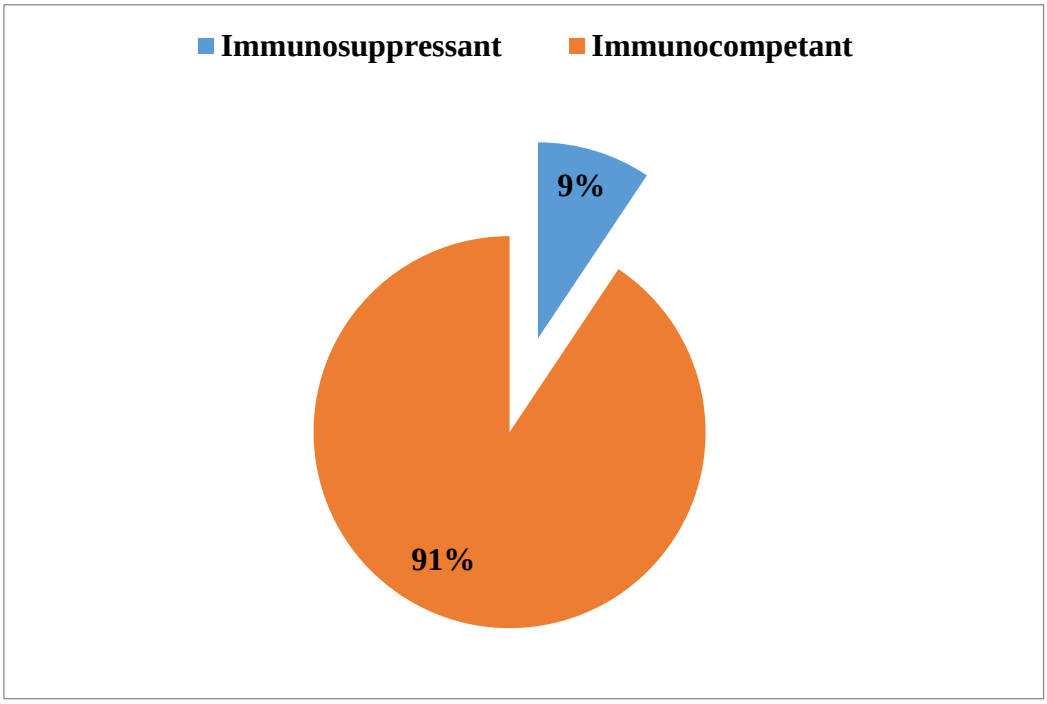


CHART 8: CYTOLOGY

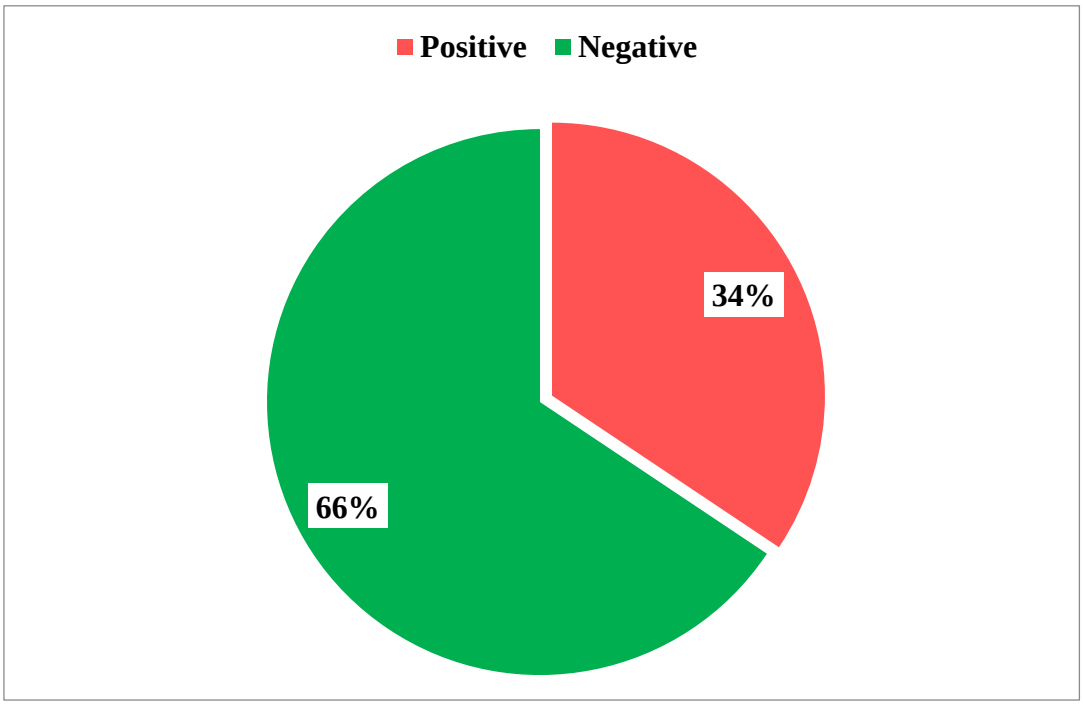


CHART 9: AFB SMEAR – ZIEHL NEELSEN TECHNIQUE

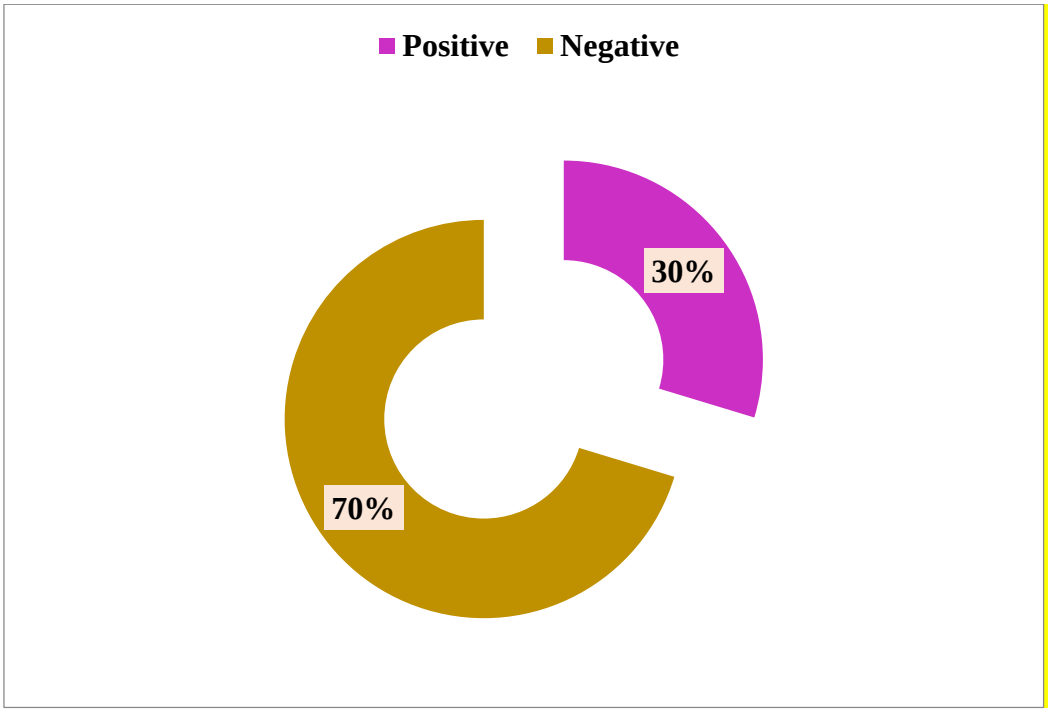


CHART 10: MOLECULAR METHOD BY PCR

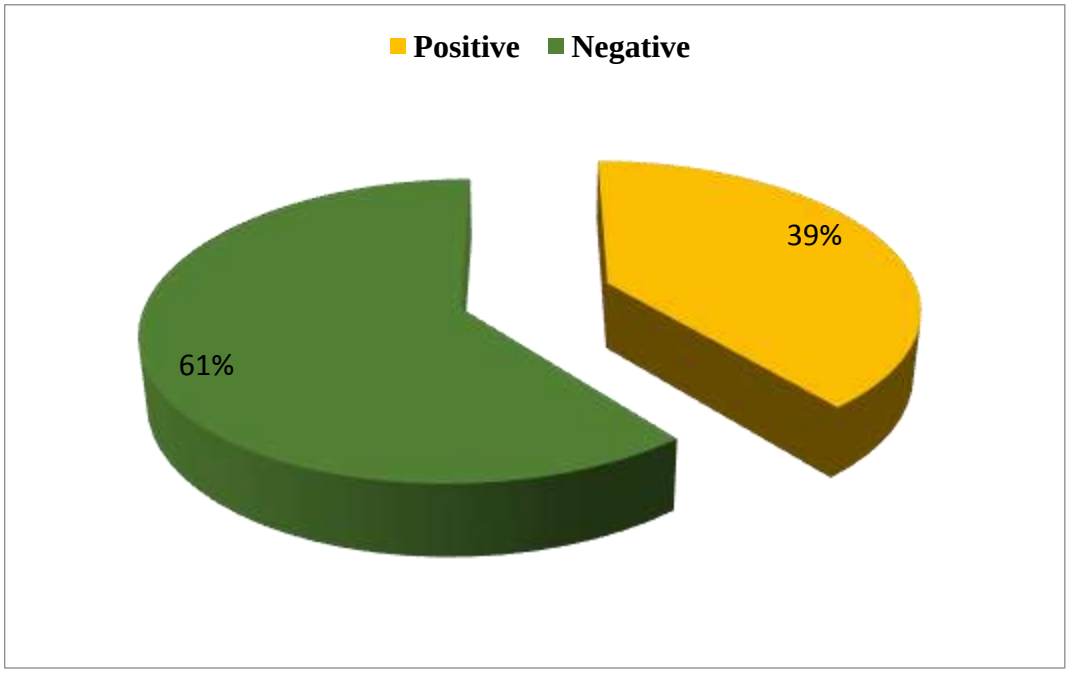


CHART 11: MOLECULAR METHOD BY GENE XPERT

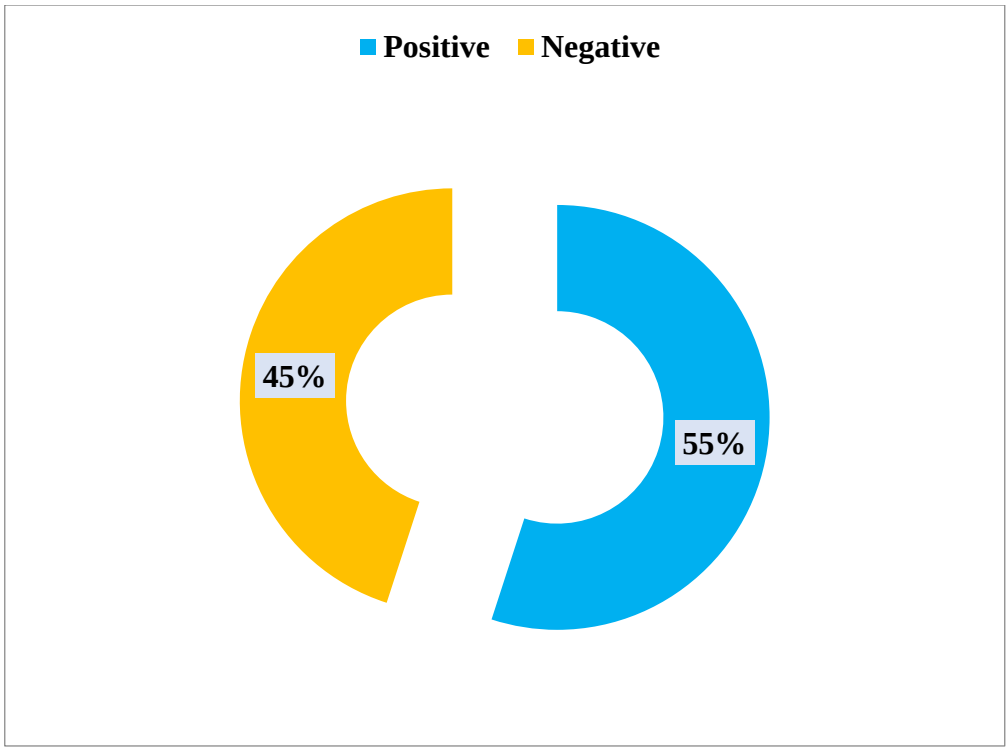


CHART 12: COMPARISON OF DIFFERENT NON-CULTURE METHODS WITH PCR

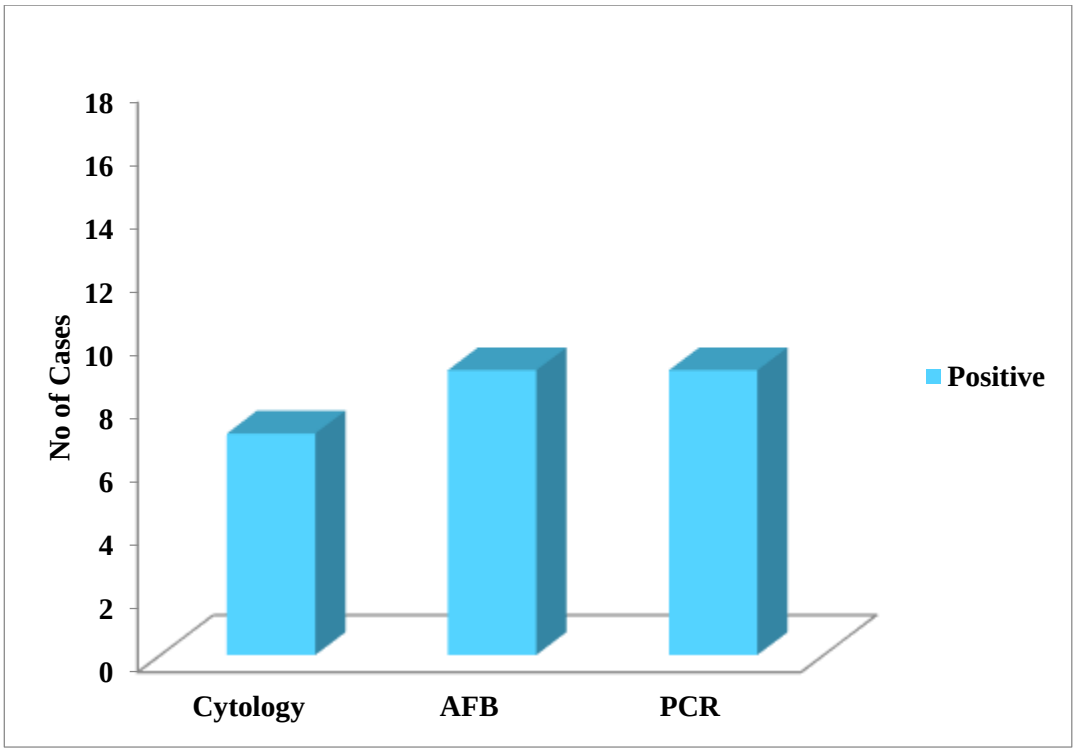
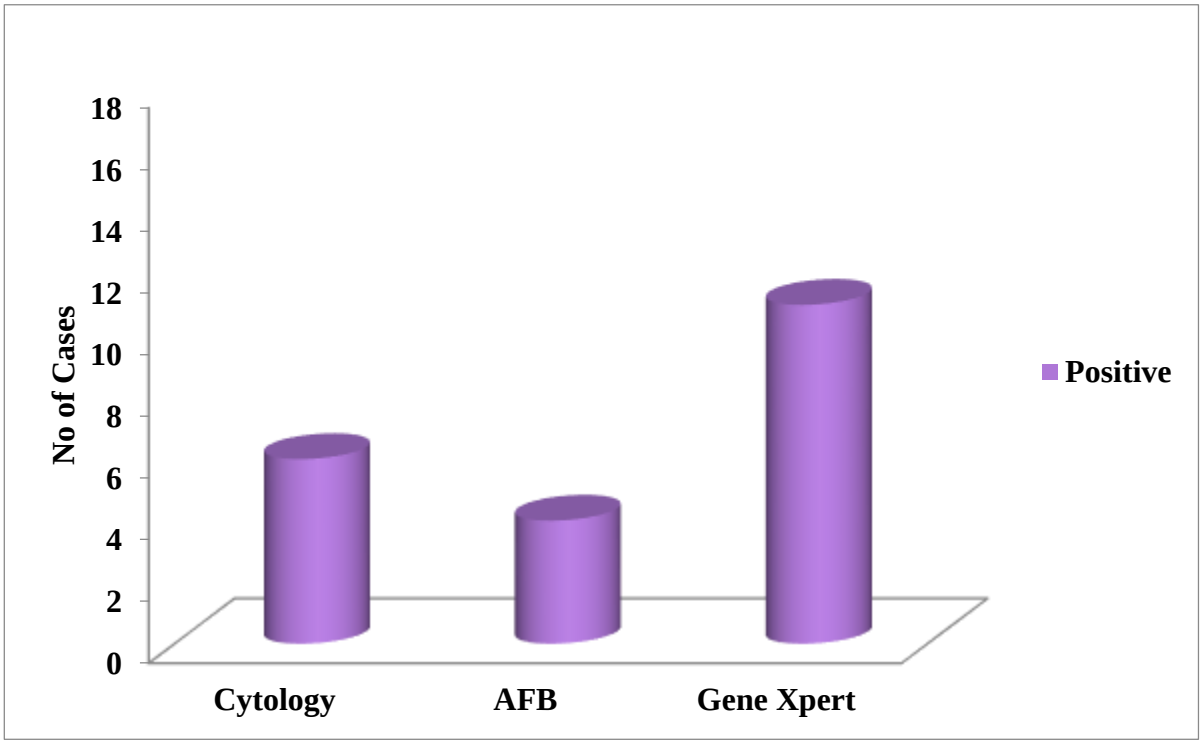


CHART 13: COMPARISON OF DIFFERENT NON-CULTURE METHODS WITH GENE XPERT



DISCUSSION

DISCUSSION

In the present study various diagnostic methods for non culture techniques including molecular methods like PCR were done from fine needle aspirates to confirm the diagnosis of tuberculous lymphadenitis and the results were compared to find out which of the techniques were more sensitive and specific and gives earlier results. Earlier results enables us to provide earlier recovery which is very important in curtailing its spread.

Among the clinically suspected tuberculous lymphadenitis cases, the sex distribution in this study showed that there was a mild female preponderance, the ratio with male being 1.2:0.87. This study is similar to study conducted in tertiary hospital VMMC and Safdarjung Hospital, New Delhi which also showed female preponderance.

This study showed that tuberculosis lymphadenitis is most frequently found in the age group between 15-30 yrs., which consisted of the productive age group and is similar to the study conducted by Dhawan et al, in Safdarjung Hospital.

From this study, we can observe only 16 out of 64 patients showed typical history whereas remaining 48 patients presented without constitutional symptoms and non-specific complaints makes delay in suspecting the disease. Out of 16 patients showing typical history, 13 (81%) patients were found to be positive and out of 48 patients without typical symptoms ,19 (40%) patients were found to be positive .

It was observed in this study the most commonly involved site of lymphadenopathy was posterior triangle of cervical nodes followed in the descending order of frequency by anterior triangle of cervical nodes, supraclavicular and submandibular lymph nodes. This observation is similar to the study of Bibhuti Das et al.

In this study only 9 samples were purulent whereas remaining 55 samples were minimally fluid with or without blood. Out 9 purulent samples, 7 samples were found to be positive and out of remaining 55 non purulent (minimal fluid) samples, 33 samples were found to be positive. Obtaining the adequate sample other than fluctuant modes is quite difficult for which the molecular study has been restricted to selected samples.

In the present study out of 64 samples , 19 cases were positive for AFB by Ziehl Neelsen method . On comparing with PCR, the sensitivity of the smear AFB was 66.67%, specificity was 78.57% and when comparing with GeneXpert the sensitivity was 39.36% and specificity was 100%. The variability of the above parameters is due its paucibacillary nature of the disease as the smear needs 10^4/ml of organism to show positive in the direct smear.

In further analysis, three smear positive cases were negative in PCR which could be due to either non-tuberculous bacterial infection (2) or due to the possibility of damage of tuberculous DNA during freezing and thawing process in RT PCR.

When compared with 2 assays in molecular methods the positive predictive value of AFB ranges from 66.67% to 100% and negative predictive value of 56.25% to 78%.

Criteria for diagnosing of Tuberculous were the cytomorphological FNA cytology showing epitheloid cells granulomas with or without multinucleated giant cells and caseation necrosis. In case of cytology showing necrosis only/non-caseating granulomas/acute suppurative lymphadenitis without demonstration of AFB were considered as suspicious of tuberculosis.

In our study, clear cut cytomorphological features obtained in 22 samples out of 64 samples. Whereas cytomorphological features suspicious of tuberculous accounts for 34 samples and remaining 8 samples were reactive lymphadenitis which clearly depicts negative status of the samples and P value (0.049) correlates with the observation .

Out of 34 suspicious sample, 12 AFB smear were positive adding real importance in diagnosis of extra pulmonary tuberculosis even though it is paucibacillary condition.

In further analysis, out of 7 cytology positive samples 3 samples were positive with PCR and 4 samples were negative and may be due to paucibacillary condition or damage to DNA due to freezing and thawing procedure. But PCR showed 6 positive samples where cytology were negative.

With Genexpert out of 6 positives ,only 2 samples for cytology were positive and 4 samples for cytology were negative may be pointing towards paucibacillary condition.

But GeneXpert showed 9 positive results where cytology proved negative.

Two assays in molecular methods were adopted as instructions were given to RNTCP to perform CB NAAT for extrapulmonary samples also only in the late phase of the study.

As GeneEpert requires fresh samples, only fresh samples were tested for CBNAAT and the remaining collected samples were subjected to realtime PCR (23 samples).

As earlier discussed due to the difficulty in obtaining the sample from nonfluctuant nodes, only 42 samples were subjected to molecular methods.

In RT PCR, except one sample out of 9 positive sample other sample had Ct value >35 and were considered as weak positives;

This may be due to ;

1. Lack of adequate sample and apportioning of the sample for various diagnostic test (cytology, microbiology, PCR) resulting in non uniform distribution of organisms.
2. Paucibacillary
3. Presence of inhibitors
4. Damage to DNA due to freezing and thawing procedure.

When compared GeneXpert with RT-PCR, GeneXpert is more sensitive as fresh samples are used. IN addition simultaneous detection of rifampicin resistance, thereby helping to start appropriate therapy which is major cornerstone in controlling the disease which is the objective of this study.

For the above reasons WHO endorsed CBNAAT even for extrapulmonary samples and CBNAAT is mandatory for tuberculous lymphadenitis along with smear microscopy, cytology, culture. In our study GeneXpert had detected a resistance pattern and it has helped the patient to get appropriate treatment which in turn helps to prevent the spread of MDR TB.

Disadvantage in GeneXpert is the possibility of missing cases as the software in Genexpert is designed to give report for cycle threshold Value upto 35 cycles are not interpreted and thereby chances of missing drug resistance cases especially in extrapulmonary sample where it is paucibacillary in nature also lack of adequate sample.

The data of the study is grouped into three. In the 1st group PCR is taken as the gold standard and compared with cytology and AFB. In the 2nd group Genexpert is taken as the gold standard and compared with cytology and AFB. In the last group AFB is taken as the golden standard and compared with cytology. The results are presented in the table.

Youden's index is used for the evaluation of overall discriminative power of a diagnostic procedure and for comparison of this test with other tests. Youden's index = (sensitivity + specificity) – 1

For a test with poor diagnostic accuracy, Youden's index equals 0, and in a perfect test Youden's index equals 1. Youden's index is not affected by the disease prevalence, but by the spectrum of the disease.

Youden's index is close to zero for cytology when compared with the other tests indicating its limitations. Association of AFB with both PCR and GeneXpert is statistically significant with 'p' values 0.03 and 0.043 respectively (Chi-squared test).

Sensitivity = (TP/TP+FN) and to recognise subjects with the disease. Specificity = (TN/TN+FP) and describes the test ability to recognise subjects without the disease.

Cytology and AFB shows high specificity in comparison with PCR (Sp: 71% and 78%) describing the tests ability to recognise the subjects without the disease.

Cytology shows very poor sensitivity on comparison with GeneXpert Se: 18.18%) describing its inability to recognise subjects with the disease. It also demonstrates the superiority of GeneXpert (as its compared with cytology).

SUMMARY

SUMMARY

Laboratory diagnosis of tuberculous lymphadenitis by non culture methods like AFB smear microscopy, cytology and molecular study were performed using fine needle aspirate samples from Coimbatore Medical College, Coimbatore.

Out of 64 samples, AFB was demonstrated in direct smear in 19 (30%) patients, cytopathological features of tuberculous lymphadenitis was reported in 22 patients (34%) samples. Out of 23 samples subjected to RT PCR targeting IS6110 fragment of DNA of mycobacterium tuberculosis, 9 samples (39.13%) were positive.

Out of 20 samples subjected to GeneXpert detecting rpoB gene and its mutation to identify the resistance of mycobacterium tuberculosis, 11 (55%) samples were found to be positive and detected mutated gene in one sample helping the patient to get appropriate treatment and prevented its spread as the patient had discharging wound..

The lab diagnosis of tuberculous lymphadenitis by molecular methods are completed in one day that too by GeneXpert within 2 hours when compared to minimum of 24 days required for detection by conventional culture method.

This study proved that by using non culture methods it is possible to get a good yield of M.tuberculosis from the paucibacillary extrapulmonary tuberculosis.

The logistic regression analysis revealed that AFB is significantly associated with PCR ($p<0.03$) and Genexpert ($p<0.043$) and found to be statistically significant. And AFB has got better predictive value than cytology.

9% of suspected were co-infected with HIV (known patients) and were showing 66% positive for tuberculous lymphadenitis and 66% of them were males ranging from between 25 – 45 years of age, the most economically productive age group.

LIMITATIONS

This study has limitations;

i) To obtain an adequate sample from non-fluctuant nodes.

ii) 2 molecular assays cannot be performed in the same sample.

CONCLUSION

CONCLUSION

- In diagnosis of tuberculous lymphadenitis (extrapulmonary) single diagnostic test is not sufficient.
- AFB has got better productive capability of tuberculous lymphadenitis compared with cytology technique and is found to be statistically significant.
- Demonstration of AFB in direct smear ($p<0.03$-0.04) of the fine needle aspirate has significant statistical associated with molecular methods.
- FNAC is a major support in case of extrapulmonary samples which is paucibacillary in nature.
- Among molecular methods Genexpert is more advantages as it defects simultaneously MDR cases also and requires very short span of time for report (2 hrs).
- There is not risk of contamination and also biosafety facilities are not needed as it is a closed system.
- RT-PCR picks up weak positive samples also when cycle threshold value is >35 which were missed by GeneXpert as it reports only up cycle threshold value 35.
- In case of extrapulmonary tuberculosis, each non-culture method contributes and increases overall sensitivity and specificity in diagnosing the condition within short span of time which is essential for TB control.

- Kit with increase sensitivity and simultaneously drug susceptibility test for more drugs is needed so that it can contribute to faster treatment of TB patients.
- In this study, molecular methods proved to be superior especially GeneXpert with its simultaneous advantage of detecting rifampicin resistance is a great adjunct to non-culture methods like AFB smear study and cytology. It is more sensitive and specific and gives earlier results.

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ANNEXURES

PROFORMA

Name :

Age :

Sex :

Mobile no :

Address :

History of DM/HT/BA/TB/others:

History of smoking/ Alcohol intake:

Past Medical History :

Past Surgical History :

Family History :

Height :

Weight :

BMI :

Pulse :

B P :

Nature of work (outdoor or indoor):

Chest X-Ray :

Sputum for AFB :

STATEMENT OF CONSENT

I, _____, do hereby volunteer and consent to participate in this study being conducted by Dr.R.PATHMINI , I have read and understood the consent form (or) it has been read and explained to me thoroughly. I am fully aware of the study details as well as aware that I may ask questions to him at any time.

Signature / Left Thumb Impression of the patient

Station: Coimbatore

Date:

Signature / Left Thumb Impressionand Name of the witness

Station: Coimbatore

Date:

STATEMENT OF CONSENT

I, _____, do hereby volunteer and consent to participate in this study being conducted by Dr. R.PATHMINI. I have read and understood the consent form (or) it has been read and explained to me thoroughly. I am fully aware of the study details as well as aware that I may ask questions to him at any time.

Signature / Left Thumb Impression of the patient

Station: Coimbatore

Date:

Signature / Left Thumb Impression and Name of the witness

Station: Coimbatore

Date:

MASTER CHART

S.No	Name & Pathology No	Age	Sex	Type of Sample	Site of Location								Cytology	AFB Smear	PCR	Gene Expert	Immunity Status	Tytpical History
					Right				Left									
					PT	AT	SB	SC	PT	AT	SB	SC						
1	Anuradha F-50/18	37	F	Pus	✓								-	+	+	+	-	+
2	Panneerselvi F-47/18	36	F	Aspirate					✓				+	-	-	NA	-	-
3	Prabhu F-2643/17	32	M	Aspirate					✓				+	-	-	NA	-	-
4	Chaitakumar F93/18	23	M	Aspirate					✓				+	-	+	NA	-	+
5	Sumitra F-130/18	19	F	Pus					✓				-	+	+	NA	-	+
6	Vaideeshwari F-161/8	22	F	Aspirate	✓								+	+	-	NA	-	+
7	Padmavathy F-279/18	65	F	Aspirate		✓							-	-	-	NA	-	-
8	Roja F-277/18	23	F	Aspirate	✓								-	+	+	NA	-	+
9	Princy F-299/18	23	F	Aspirate	✓								-	-	-	NA	-	-
10	Selvi F-304/18	37	F	Aspirate	✓				✓				+	+	-	NA	-	-
11	Sapnabanu F-328/18	23	F	Aspirate		✓							-	-	-	NA	-	-
12	Dhanalakshmi F-432/18	19	F	Aspirate		✓							-	+	-	NA	-	+
13	Subashilenka F-462/18	25	M	Pus				✓					+	+	+	NA	-	-
14	Manimala F-491/18	21	F	Aspirate		✓							-	-	-	NA	-	-
15	Ramasamy F-499/18	59	M	Aspirate		✓							-	-	-	NA	-	+
16	Kasinathan F-554/18	21	M	Aspirate					✓				+	-	+	NA	-	-
17	Jeyalakshmi F-590/18	45	F	Pus				✓					-	-	-	NA	-	-
18	Dhanalaskhmi F-619/18	51	F	Aspirate									-	-	-	NA	-	-
19	Ramu F-649/18	30	M	Aspirate	✓								-	-	-	NA	-	-
20	Soundarya F-2509/17	29	F	Aspirate	✓								-	-	+	NA	-	-
21	Aaayesha F-494/18	25	F	Pus	✓								-	+	+	NA	-	-
22	Suguna F-98/18	34	F	Pus					✓				-	+	+	NA	-	-

23	Sarmika F-642/18	22	F	Aspirate					✓				-	-	-	NA	-	-
24	Rameeladevi F-546/18	44	F	Aspirate		✓							+	-		-	-	-
25	Muthukumar F-620/18	30	M	Pus					✓				-	+		+	-	-
26	Muthukumarasami F-668/18	36	M	Aspirate	✓		✓						-	+		+	+	+
27	Ramesh F-627/18	28	M	Aspirate					✓				+	-		+	+	+
28	Mukeshkaor F-766/18	28	F	Aspirate	✓				✓				-	-		+	-	-
29	Vijayalakshmi F-780/18	22	F	Aspirate				✓					-	-		-	-	-
30	Rasu F-805/18	33	M	Aspirate	✓								-	+		+	-	-
31	Shankar F-811/18	27	M	Aspirate									+	-		+	-	+
32	Madesh F-842/18	43	M	Aspirate									+	-		-	+	-
33	JyotiShabarek F-877/18	20	M	Aspirate					✓				-	-		+	-	-
34	Muthuraj F-898/18	23	M	Aspirate				✓					-	-		+	-	-
35	Amudha F-1102/18	42	F	Aspirate		✓							-	-		-	+	-
36	Padndiammal F-1120/18	44	F	Aspirate				✓					+	-		-	-	-
37	Savithri F-1163/18	27	F	Aspirate				✓					-	-		+	-	-
38	Vengadesh F-1244/18	26	M	Aspirate		✓				✓			-	-		+	-	-
39	Kavitha F-1338/18	45	F	Aspirate				✓					-	-		-	-	-
40	Gomathi F-1403/18	30	F	Aspirate	✓								-	-		-	-	-
41	Rathinal F-1383/18	46	F	Aspirate					✓				+	-		-	-	-
42	Saranya F-1397/18	28	F	Aspirate					✓				-	-		-	-	-
43	Priya 530603	30	F	Pus						✓			-	-			-	+
44	Pongodi 1273401	21	F	Aspirate	✓				✓				+	+			-	+
45	Mahendran 1273702	54	M	Aspirate				✓					+	+			-	-
46	Vijaya 468953	40	F	Aspirate	✓								-	-			-	-
47	Anwarali 1281459	35	M	Aspirate									-	-			-	-

48	Murugeshwari 1281459	19	F	Aspirate								✓	+	-			-	-
49	Mega 1256222	22	F	Aspirate		✓							+	-			-	+
50	Sarasu 1290401	50	F	Aspirate				✓					-	-			-	-
51	Vinith 1295594	24	M	Aspirate	✓								+	-			-	-
52	Boopathi 1294075	29	M	Aspirate	✓								+	-			-	-
53	Pushpalatha 1295168	27	F	Aspirate					✓				+	+			-	-
54	Palanisamy 7835/17	44	M	Aspirate		✓							-	-			+	-
55	Deivanai F-226/18	44	F	Pus				Axilla				Axilla	-	+			+	-
56	Anand 18	27	M	Aspirate	✓								-	+			-	+
57	Sokkalingam F-536/18	56	M	Aspirate	✓								-	-			-	+
58	Sathyavani F-687/18	58	F	Aspirate		✓							-	-			-	-
59	Katheeswari F-688/18	26	F	Aspirate						✓			-	-			-	-
60	Chandru F-693/18	30	M	Aspirate					✓				-	-			-	-
61	Kanmani F-694/18	35	F	Aspirate	✓	✓							-	+			-	-
62	Vigneshkumar F-703/18	23	M	Aspirate	✓								+	-			-	-
63	Keerthana F-717/18	15	F	Aspirate	✓								+	+			-	+
64	Kanniyammal F-718/18	70	F	Aspirate	✓								-	-			-	-

PT :Posterior Triangle **AT** :Anteior tTriangle **SB** : Submandibular **SC** :Supraclavicular